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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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SKIN INFECTION OF RABBITS WITH HEMOLYTIC STREPTOCOCCI ISOLATED FROM A PATIENT WITH ERYSIPELAS.

I. Method of Demonstrating Protective Action of Immune Sera.

By THOMAS M. RIVERS, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plate 9.

(Received for publication, September 10, 1924.)

The present paper deals with the lesions produced in the skin of rabbits by means of a hemolytic streptococcus isolated from a human case of erysipelas, and with the preparation of an immune serum capable of protecting the skin against the injurious action of this organism. Previous observers have described the lesions produced by the injection of streptococci into the skin of animals and man. In most of the experiments in animals the injections have been made into the skin covering the ear; and in certain instances a local spreading lymphangitis, resembling erysipelas, has been produced. In man, typical erysipelas has resulted from the injection of hemolytic streptococci made for therapeutic purposes. The lesions which have been described as developing in animals after injections of streptococci have been of several types and in the past writers have not always been careful to distinguish between them. No attempt will be made to review the voluminous literature on the experimental production of erysipelas or streptococcal lesions in the skin as this has been done recently by Gay.[1,2]

Method of Inoculation.

The organism employed in all the experiments to be reported was isolated from a human case of erysipelas. In this case, on account of the development

[1] Gay, F. P., and Rhodes, B., *J. Infect. Dis.*, 1922, xxxi, 101.

[2] Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1923, xxxiii, 338.

of a deep infiltration 10 days following the onset, an incision into the skin was made for therapeutic purposes and from the fluid which escaped the streptococcus was isolated. The organism is morphologically and culturally typical of the class of hemolytic streptococci. It is not highly virulent for rabbits on intravenous or intraperitoneal inoculation,—2 cc. of an 18 hour broth culture failing to kill. The organism has been kept as nearly as possible in its original state by an occasional intracutaneous passage through rabbits.

Suspensions in 8 cc. of Locke's solution of the bacteria removed from a 24 hour culture on a blood agar slant were employed for making the inoculations. After the suspensions had been shaken thoroughly various dilutions were made as desired. By means of a tuberculin syringe and a 22 gauge needle, 0.1 cc. of each dilution was injected intracutaneously into rabbits shaved over the sides of the chest and abdomen. Although an effort was always made to prevent any of the inoculum escaping into the subcutaneous tissues, it is not unlikely that in some instances this happened to some extent. Frequently fifteen or twenty intracutaneous inoculations made at one time in a single animal were survived. Rabbits vary somewhat in susceptibility to skin infections with streptococci. Hence a comparison between the results of numerous inoculations upon a single individual yielded results more readily interpretable than would have been the case had they been made on different animals.

Description of Skin Lesions.

Marked reactions appeared about the sites of injection of the heavier suspensions of streptococci. The severity of these reactions decreased with diminishing concentrations of the inoculum (Fig. 1); and usually no lesion was visible when the dilution of the original bacterial suspension was greater than 1:500. Within 24 to 48 hours the larger lesions, 3 to 4 cm. in diameter, were red, hot, and edematous. Frequently the skin over the center of the lesions was white or purple (Fig. 1). The lesions increased in size and severity and often spread ventrally in the form of red edematous bands. The centers of the lesions became necrotic, and scabs formed, beneath which pus collected. The lesions healed slowly, often requiring a month to do so. The milder ones frequently disappeared without becoming necrotic. This type of lesion was considered as phlegmons or abscesses and was the usual kind of reaction to follow the inoculations of the streptococcus employed in these experiments.

Besides the phlegmons, in approximately one of every ten rabbits inoculated, another type of reaction occurred. In these instances the skin appeared red and somewhat swollen outside of the phleg-

monous areas. The reaction extended in all directions, dorsally as well as ventrally, and the advancing margin of the lesion was irregular and slightly raised. Near the margin the skin was bright red, while over the more central portions it was paler. Not infrequently such lesions spread to portions of the skin where the hair had not been removed. When the animal had received several inoculations, this type of reaction usually occurred around only one of the phlegmonous areas. The spreading of the inflammation usually continued 6 or 7 days and then the acute reaction subsided rapidly and was followed by a marked desquamation of the skin involved. The central phlegmonous area, however, persisted for some time. This second type of lesion had many of the characteristics which one associates with human erysipelas.

Preparation of Immune Serum.

An attempt was made to produce an immune serum by repeated inoculations of streptococci into rabbits, not as is usually done by injecting the bacteria into the blood or peritoneal cavity, but by intracutaneous inoculations.

Seven rabbits were inoculated intracutaneously in a single area with 0.2 cc. each of a suspension of the streptococci. In each rabbit a large phlegmonous lesion resulted. 10 days later each of the rabbits received a second injection. The lesions following the second inoculations were smaller than those previously observed. After 7 days the rabbits received a third inoculation. The lesions resulting from these inoculations were small, reached their maximum size in 24 to 48 hours, and frequently disappeared without breaking down. Four more similar inoculations were made at weekly intervals. Following these later injections the reactions were mild. Care was taken not to inoculate the same area of the skin more than once. 8 days after the seventh injection the rabbits were anesthetized and bled from the heart. The sera were collected, pooled, inactivated, and stored on ice. Seven normal rabbits were bled as well. Their sera were pooled and stored to be used for control purposes.

Demonstration of the Protective Properties of the Immune Serum.

To determine whether the serum prepared as described possessed any protective or immunizing properties the following method was employed.

A series of dilutions of a streptococcus suspension was made. Two portions of each dilution were taken; to one portion an equal quantity of the immune serum was added, and to the second portion an equal quantity of the normal serum. 0.1 cc. of these mixtures was injected immediately into the shaved skin of the same rabbit. On one side of the animal, mixtures of the immune serum and dilutions of the streptococcus suspension were inoculated, on the other side a similar series of injections was made with mixtures of normal serum and dilutions of the suspension of organisms.

At the sites of inoculation of the mixtures of immune serum and streptococci only a slight redness was seen 2 days after the injections (Fig. 2). On the opposite side of the rabbit, however, where mixtures of normal serum and streptococci had been injected, large lesions developed similar to those occurring after inoculations of streptococci alone (Fig. 1). 7 days after the inoculations the sides of the rabbit exhibited a very striking contrast. On that inoculated with mixtures of immune serum and streptococci only small nodules without necrotic centers were seen (Fig. 4). On the opposite side (Fig. 3) large nodules filled with pus and covered by scabs were present. This experiment has been repeated many times and the results were always just as striking as those described. Always at the sites where normal serum plus streptococci were injected large lesions appeared. Inoculations of streptococci plus serum from treated animals were followed by little if any reaction. The evidence was convincing that a true immune serum capable of warding off the injurious action of the streptococci on the skin had been developed in response to repeated intracutaneous inoculations of the organism.

DISCUSSION.

The reasons why in some animals and at some situations an erysipelas-like lesion developed, not the ordinary phlegmon, have not yet become apparent. It seems quite evident, however, that some-

thing more than the mere intradermal injection of the hemolytic streptococcus in a shaved rabbit is necessary for the development of such a lesion. This problem deserves and will be given further study.

Should the method here described for demonstrating the immune properties of antistreptococcus serum prove of general applicability, it will be found to possess a number of advantages. To determine the protective action of an antibacterial serum by intraperitoneal injection, as for instance in testing antipneumococcus serum, requires that the organism employed be maintained at a constant and high level of virulence. This is diffcult or impossible in the case of streptococci as is well known. Tests of the protective action of sera by *in vitro* methods, as by agglutination or precipitation, are recognized as being of doubtful value in most cases. It is possible that the method may be of value not only to determine the potency of sera for therapeutic purposes but for tests of the specificity of different strains of the streptococcus and for the solution of certain obscure facts concerning immunity to streptococci.

SUMMARY.

Cultures of a hemolytic streptococcus isolated from a human case of erysipelas were tested for ability to produce lesions by injecting various amounts intradermally in shaved rabbits. Severe lesions could be developed at will in this way despite the fact that intravenous or intraperitoneal inoculations of large doses of the cultures failed to kill. The lesions were of two sorts, phlegmonous and erysipelas-like.

A serum capable of warding off the injurious action of the streptococcus on the skin was procured from animals receiving repeated intracutaneous inoculations of the organism. Normal serum by contrast had no protective effect.

By intradermal injections at several points in the same normal rabbit of small quantities of mixtures of equal parts of serum and various dilutions of a suspension of streptococci it proved possible to test sera for the presence of substances protective against the streptococcus.

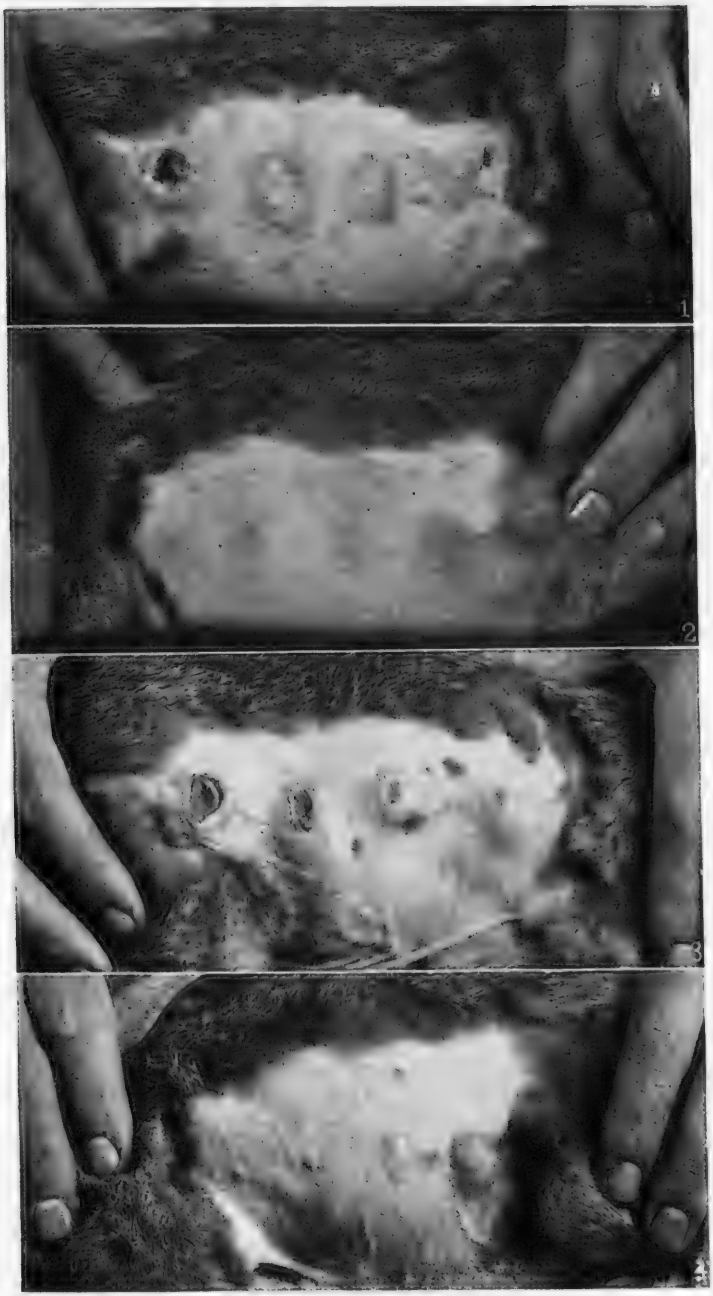
EXPLANATION OF PLATE 9.

FIG. 1. Lesions on the right side of Rabbit A 48 hours after the intracutaneous injection of 0.1 cc. of mixtures of equal parts of normal serum and various dilutions of a streptococcus suspension. The doses of streptococci were decreased from left to right.

FIG. 2. Lesions on the left side of Rabbit A 48 hours after the intracutaneous injection of 0.1 cc. of mixtures of equal parts of immune serum and various dilutions of a streptococcus suspension. The doses of streptococci were decreased from right to left.

FIG. 3. Same as Fig. 1, 5 days later.

FIG. 4. Same as Fig. 2, 5 days later.



(Rivers: Erysipelas. I.)

LOCAL PASSIVE IMMUNITY IN THE SKIN OF RABBITS TO INFECTION WITH (1) A FILTERABLE VIRUS, AND (2) HEMOLYTIC STREPTOCOCCI.

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PLATES 10 AND 11.

(Received for publication, September, 10, 1924.)

Observations reported in a previous paper[1] concerning the neutralization of a filterable rabbit virus by immune serum have led us to experiments on the production of local passive immunity to this virus. The experiments have been instrumental in arousing our interest in the general subject of local passive immunity. A report on them will be followed in the present paper by another dealing with experiments on local passive immunity in the skin of rabbits against infections with hemolytic streptococci isolated from a human case of erysipelas.

The phenomena of local resistance and local immunity against infectious agents have interested a number of investigators for many years. Special attention has been directed recently towards the study of problems in this field by Besredka,[2] and by Gay,[3,4] who has comprehensively reviewed the literature.[5] In the treatment of certain infectious diseases the advantages pertaining to the local administration of the antisera are well known. These advantages have been enumerated and emphasized by Flexner.[6]

Significant observations, not included in Gay's[5] review, concerning local passive immunity in human skin have been recorded by Blake[7] and Debré.[8] Blake states that the intradermal injection of Dochez's antiscarlet serum blanches the rash of

[1] Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1924, xxxix, 777.

[2] Besredka, A., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1923–24, xvii, 346.

[3] Gay, F. P., and Rhodes, B., *J. Infect. Dis.*, 1922, xxxi, 101.

[4] Gay, F. P., and Morrison, L. F., *J. Infect. Dis.*, 1923, xxxiii, 338.

[5] Gay, F. P., *Physiol. Rev.*, 1924, iv, 191.

[6] Flexner, S., *J. Am. Med. Assn.*, 1913, lxi, 447, 1872.

[7] Blake, F. G., Trask, J. D., Jr., and Lynch, J. F., *J. Am. Med. Assn.*, 1924, lxxxii, 712.

[8] Debré, R., Bonnet, H., and Broca, R., *Compt. rend. Soc. biol.*, 1923, lxxxix, 70,

scarlet fever patients locally and that the blanching persists. Furthermore, he states that when the serum is given intradermally during the first 24 hours of the disease, in addition to the persistent local blanching of the rash, the skin does not later become pigmented over this area nor does it desquamate. Normal horse serum, on the other hand, fails to blanch the rash. Debré reports the following observations upon the use of convalescent serum in the prevention of measles. When the serum is given soon after exposure, the disease is prevented, but when given 5 or 6 days after exposure a modified measles eruption results. Serum administered subcutaneously at the beginning of the invasion inhibits the eruption locally over the area of injection. A rash already present is not blanched by convalescent serum.

Methods.

Rabbits rendered immune to the virus above referred to were anesthetized and bled from the heart. The sera were collected, pooled, inactivated, and stored on ice. No preservative was added to the serum. The virucidal properties of the serum were tested in the manner described in a previous report.[1] Several normal rabbits were also bled from the heart. The sera were collected, inactivated, and stored on ice without the addition of a preservative. The sera from normal rabbits were tested individually for virucidal properties before they were pooled. This was necessary since it has been shown that the serum of about 20 per cent of stock rabbits neutralizes the virus.[1]

The source of the hemolytic streptococcus, the preparation of suspensions of the streptococci, and the preparation of the normal and the immune sera employed in these experiments were described in a preceding paper.[9]

Extensive areas of skin over both sides of the chest and abdomen of rabbits were shaved. By means of tuberculin syringes and 22 gauge needles areas of shaved skin about 2 cm. in diameter were infiltrated with sera, broth, or other substances as desired. Care was taken to make the infiltrations intracutaneous, yet it is not unlikely that a portion of the infiltrated substances reached the subcutaneous tissues.

Local Protection against the Action of the Rabbit Virus as Result of Injection of Immune Serum.

To determine whether the skin of rabbits can be locally protected against the action of the virus the following experiments were peformed.

Two normal rabbits were shaved on both sides of the body. On the left side of each animal an area 2 cm. in diameter was infiltrated intracutaneously with 0.5 to 0.75 cc. of immune serum. On the right side of each rabbit a similar area was

[9] Rivers, T. M., *J. Exp. Med.*, 1925, xli, 179.

infiltrated with normal serum. 24 hours later 0.2 cc. of a fresh testicular emulsion containing active virus was injected intracutaneously in each of the infiltrated areas. In addition to these inoculations a normal portion of the skin on each side, 2 inches away from the infiltrated areas, was inoculated with the virus. Two other rabbits were treated in a similar manner with the exception that 48 hours elapsed between the infiltrations with the sera and the inoculations with the virus. The animals were kept under observation during the 10 days following the inoculations.

The virus did not produce visible lesions in areas of skin infiltrated with immune serum 24 to 48 hours previously. Lesions were pro-

TABLE I.

Summary of Experiments Demonstrating in the Skin of Rabbits a Local Passive Immunity to Virus III.

Rabbit No.	Left side of rabbit.		Right side of rabbit.	
	Area of skin infiltrated with immune serum.	Uninfiltrated area of skin.	Area of skin infiltrated with normal serum.	Uninfiltrated area of skin.
Virus injected 24 hrs. after the skin was infiltrated with the sera.				
1	−	+	+	+
2	−	+	+	+
Virus injected 48 hrs. after the skin was infiltrated with the sera.				
3	−	+	+	+
4	−	+	+	+

+ indicates the presence of a visible lesion usually produced in the skin of rabbits by the virus. − indicates absence of a visible lesion.

duced by the virus, however, in areas of skin not so infiltrated and essentially similar ones also in the areas of skin infiltrated with normal serum. No titrations of the virus were made to determine whether, despite these findings, a slight degree of protection had resulted from the infiltrations of normal serum. The results of the experiments are summarized in Table I.

Since intracutaneous infiltrations of normal serum did not and intracutaneous infiltrations of immune serum did inhibit the action of the virus in the same animals, it seems reasonable to conclude that a local passive immunity against the action of the virus was produced

in the skin of rabbits by the infiltrations of immune serum. This local protection persisted for at least 48 hours.

Local Protection against the Action of Hemolytic Streptococci as Result of the Injection of Broth, Normal Serum, or Immune Serum.

A review of the literature on hemolytic streptococci seemed to indicate that meat infusion broth and possibly normal serum infiltrated into the skin might produce some local protection against these organisms injected 24 hours later. It was necessary to determine the truth of the matter before attempting to induce a local passive immunity. The experiments with broth and with normal serum will be described first and then the experiments with immune serum will be reported.

Broth.—The following experiment was performed to determine whether intracutaneous infiltrations of broth give rise to local protection in the skin of rabbits against the action of hemolytic streptococci.

Two rabbits were shaved on both sides of the body. In four areas on the right side of each animal, 0.5 cc. of meat infusion broth was infiltrated intracutaneously. The left side was merely shaved. 24 hours after the infiltrations, various dilutions of a streptococcus suspension were injected intracutaneously to the amount of 0.1 cc. in four areas on each side of both rabbits. The inoculations on the right side were made in the center of each area previously infiltrated with broth. The animals were examined each day for 10 days.

No lesions developed in the areas of skin infiltrated with broth 24 hours prior to inoculation with streptococci. A definite lesion developed at the point of each inoculation on the uninfiltrated side (Figs. 1 and 2). The experiment demonstrated conclusively that meat infusion broth infiltrated into the skin of a rabbit protected it 24 hours later against doses of hemolytic streptococci that produced visible lesions in the uninfiltrated skin of the same animal. The results of the experiment are summarized in Table II.

It was found absolutely necessary to infiltrate the skin before the streptococci were inoculated if protection was desired because broth was found to afford no protection when mixed with the suspensions of streptococci and then injected into normal skin. Data are not available at the moment with regard to how soon the protection

appears after the infiltrations or how long it persists. 24 hours was chosen arbitrarily as the period to elapse between the broth infiltrations and the streptococcus inoculations.

Normal Rabbit Serum.—The details of the experiments with normal rabbit serum will not be given because normal serum was used as a control in all of the experiments performed with immune serum and abundant opportunities will be presented in these to observe its effects. It is sufficient to say that infiltrations of 0.5 cc. of normal rabbit serum into areas of skin were found to afford considerable local protection against infection with hemolytic streptococci inoculated 24 hours later. No experiments were made to compare the effectiveness of broth with that of normal serum. The impression was

TABLE II.

Summary of an Experiment Showing that Meat Infusion Broth Infiltrated in the Skin of Rabbits Affords Local Protection against Infection with Hemolytic Streptococci Inoculated 24 Hours Later.

Rabbit No.	Right side of rabbit infiltrated in each of four areas with 0.5 cc. of meat infusion broth 24 hrs. previous to inoculation with the streptococci.				Left side of rabbit unhandled except for shaving.			
	Dilutions of streptococcus suspension.				Dilutions of streptococcus suspension.			
	1:4	1:8	1:16	1:32	1:4	1:8	1:16	1:32
1	−	−	−	−	+	+	+	+
2	−	−	−	−	+	+	+	+

+ indicates presence of visible lesion at site of inoculation of the streptococci.
− indicates absence of visible lesion.

obtained, however, that infiltrations of the broth afforded more protection than did infiltrations of similar amounts of normal serum. As with broth, the normal serum had to be infiltrated into the skin some hours before the streptococci were inoculated if protection was to be obtained.

Immune Serum.—No attempt has been made to analyze the mechanism of the protection afforded by normal serum and broth which, for convenience, will be spoken of as a local non-specific protection. It now seemed desirable to determine if a specific local passive immunity against hemolytic streptococcus infections could be demonstrated in areas of skin infiltrated with a homologous immune serum 24 hours

prior to inoculation. Therefore experiments were performed to ascertain if more protection was afforded by infiltrations of immune serum than by infiltrations of normal serum. In order to do this it was necessary that each experiment should be complete in one animal and the doses of streptococci had to be so graded that an infection would occur in the areas of skin infiltrated with normal serum but not in those infiltrated with immune serum. Furthermore, one had to be sure the results obtained with immune serum were not merely the consequence of variations in non-specific protection. For this reason it was necessary to employ immune serum and normal serum from many rabbits. In the majority of instances the immune serum and the normal serum were pooled specimens from at least six or seven rabbits. Of the many experiments performed, only two will be described in detail as they furnish completely the desired information. The results all attested to the same facts.

Rabbit 1 was shaved on both sides of the body. Three areas of skin 2 cm. in diameter on the right side were infiltrated with 0.5 cc. each of serum, the front and rear areas with normal serum, the middle one with immune serum. 24 hours later the three areas were inoculated with 0.1 cc. each of the same streptococcus suspension. The left side was untouched except for shaving until 24 hours later, when it was inoculated in two places, the front one with 0.1 cc. of a mixture of equal amounts of immune serum and streptococcus suspension, the rear one with 0.1 cc. of a mixture of equal amounts of normal serum and streptococcus suspension. Portions of the same immune and normal sera were used for the tests on the two sides and the same suspension of streptococci was used throughout. The inoculations were nearly simultaneous.

It was found that immune serum and streptococci mixed and immediately injected into the skin produced no lesion. The mixture of normal serum and streptococci, however, gave rise to a large phlegmon around which an erysipelas-like lesion developed later. No lesion developed in the area of skin that was infiltrated with immune serum and inoculated with streptococci 24 hours later, whereas in those areas that had been infiltrated with normal serum definite lesions developed. They were much smaller, however, than the phlegmon that developed on the other side of the animal where the mixture of streptococci and normal serum was injected.

Rabbit 2 was shaved on both sides of the body. Four areas of skin 2 cm. in diameter on each side were infiltrated with serum in the usual manner. Pooled normal sera were used on the left, pooled immune sera on the right. 24 hours after the infiltrations the four areas on each side were inoculated respectively from back to front with decreasing doses of streptococci. The animal was examined daily for a fortnight.

No visible lesions developed following the inoculations of streptococci in the areas of skin previously prepared with immune serum.

TABLE III.

Summary of Two Experiments Showing that Immune Serum Affords More Protection than Normal Serum When Infiltrated into the Skin 24 Hours Prior to Inoculations of Hemolytic Streptococci.

Rabbit No.	Right side.				Left side.			
	Front.	Middle.	Rear.		Front.		Rear.	
1	Infiltrated with 0.5 cc. normal serum and inoculated 24 hrs. later with streptococci.	Infiltrated with 0.5 cc. immune serum and inoculated 24 hrs. later with streptococci.	Infiltrated with 0.5 cc. normal serum and inoculated 24 hrs. later with streptococci.		Skin shaved and 24 hrs. later inoculated with a mixture of streptococci and immune serum.		Skin shaved and 24 hrs. later inoculated with a mixture of streptococci and normal serum.	
	+	−	+		−		++++	
	The four places were infiltrated with 0.5 cc. each of immune serum and then 24 hrs. later were inoculated with 0.1 cc. of different dilutions of the streptococcus suspension.				The four places were infiltrated with 0.5 cc. each of normal serum and then 24 hrs. later were inoculated with 0.1 cc. of different dilutions of the streptococcus suspension.			
	1:8	1:4	1:2	Undiluted.	1:8	1:4	1:2	Undiluted.
2	−	−	−	−	++	++	++	++

+ indicates a visible lesion. The number of pluses represent the size of the lesion, ++++ being the maximum. − indicates absence of a visible lesion.

Large lesions were produced by the streptococci inoculated in the skin previously prepared with normal serum. None of these lesions, however, became necrotic even though the doses of streptococci used in the experiment regularly produced large necrotic lesions in unprotected skin.

The two experiments are summarized in Table III and the results are pictured in Figs. 3 to 8. Areas of skin prepared with normal or immune sera were more refractory to infection with hemolytic

streptococci than was unprepared skin in the same animal. Since infiltrations with immune serum always afforded more protection than that produced by infiltrations with similar amounts of normal serum, one is justified in concluding that a local passive immunity was conferred by the immune serum.

DISCUSSION.

The experiments reported in the present paper were planned for the study of local passive immunity and its value as a prophylaxis against certain local infections in the skin. As the protocols show, a local passive immunity can be induced both to a filterable virus pathogenic for rabbits and to a hemolytic streptococcus.

Previous work by other authors has shown that a non-specific refractory state against infection with hemolytic streptococci can be induced by the injection of broth and probably of many other substances. One concludes from Gay's[4] work that some of these must be administered in proper concentrations if a refractory rather than a more susceptible condition is to be obtained. Although in the present work infiltrations with normal serum produced a definite refractory state, a greater degree of local protection was conferred when immune instead of normal serum was employed. This difference in the amount of protection afforded may be thought of as representative of the degree of local passive immunity conferred by the immune serum. Further work will be required to show whether it is dependent upon the slow absorption of the serum and its protective bodies from the skin, upon a local fixation of the latter bodies altering the reactivity of the cells in the vicinity of the injected serum, or upon the mobilization of some special type of cell. Regardless of the mechanism underlying the phenomenon, it seems obvious from its occurrence that a local passive immunity can be induced and exist long enough to be of value in the prevention of certain local infections in the skin.

The question naturally arises as to whether the information obtained from the experiments here reported can be applied to the treatment of erysipelas. One might conceivably treat a local hemolytic streptococcus infection by infiltrations of the healthy tissues in the immediate vicinity with immune serum, on the assumption that when the infection spreads up to the prepared zone it will meet refrac-

tory tissues and be unable to progress further. This problem deserves and will receive further study.

SUMMARY.

Small areas of skin infiltrated with immune serum proved refractory to the injurious action of a filterable rabbit virus inoculated 24 to 48 hours later. Infiltrations of normal serum did not appreciably protect the skin against the virus. The local protection afforded by the infiltrations of immune serum appears to constitute an example of local passive immunity.

Small areas of skin infiltrated with either normal serum or meat infusion broth proved more refractory than normal skin to infection with hemolytic streptococci inoculated 24 hours later. The refractory state induced in the tissues was non-specific in character. A greater amount of local protection against hemolytic streptococci was afforded by infiltrations with a homologous immune serum than by infiltrations with normal serum. The difference in the amount of protection afforded by immune serum over that induced by normal serum may be thought of as representative of the degree of local passive immunity conferred.

EXPLANATION OF PLATES.

PLATE 10.

FIG. 1. Lesions produced by various dilutions of a suspension of hemolytic streptococci inoculated into the normal shaved skin of a rabbit. Photographed 7 days after the inoculations.

FIG. 2. Other side of rabbit of Fig. 1. Four areas of skin were infiltrated with 0.5 cc. each of meat infusion broth. 24 hours later these areas of skin were inoculated with portions of the same dilutions of the streptococcus suspension used on the side shown in Fig. 1. No lesions developed following the inoculations of streptococci into the infiltrated skin. Photographed 7 days after the inoculations.

FIG. 3. Three areas of skin were infiltrated with 0.5 cc. each of serum, the front and rear ones with normal rabbit serum, the middle one with immune serum. 24 hours later the infiltrated areas were inoculated with 0.1 cc. each of a suspension of hemolytic streptococci. No lesion developed in the skin infiltrated with immune serum. Small lesions developed in the skin infiltrated with normal serum. Compare with Fig. 4. Infiltrations of normal serum afforded some protection but not so much as that afforded by immune serum. Drawing 7 days after the inoculations.

FIG. 4. Other side of rabbit of Fig. 3. Equal parts of immune serum and a suspension of hemolytic streptococci were mixed and 0.1 cc. of this mixture was injected immediately into the normal skin. No lesion followed this inoculation. 0.1 cc. of a similar mixture of normal serum and streptococci was injected into the skin. A phlegmon followed this inoculation. Later an erysipelas-like lesion developed around the phlegmon. Immune serum protected, normal serum did not. Drawing 7 days after the inoculations.

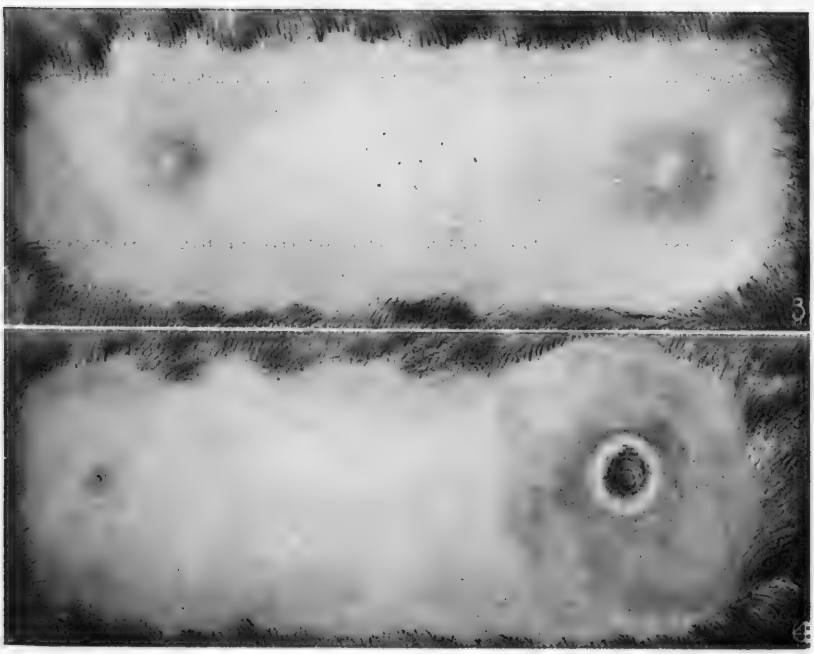
PLATE 11.

FIG. 5. Lesions produced by various dilutions of a hemolytic streptococcus suspension inoculated into four areas of skin 24 hours after they were infiltrated with 0.5 cc. of normal rabbit serum. Photographed 2 days after the inoculations.

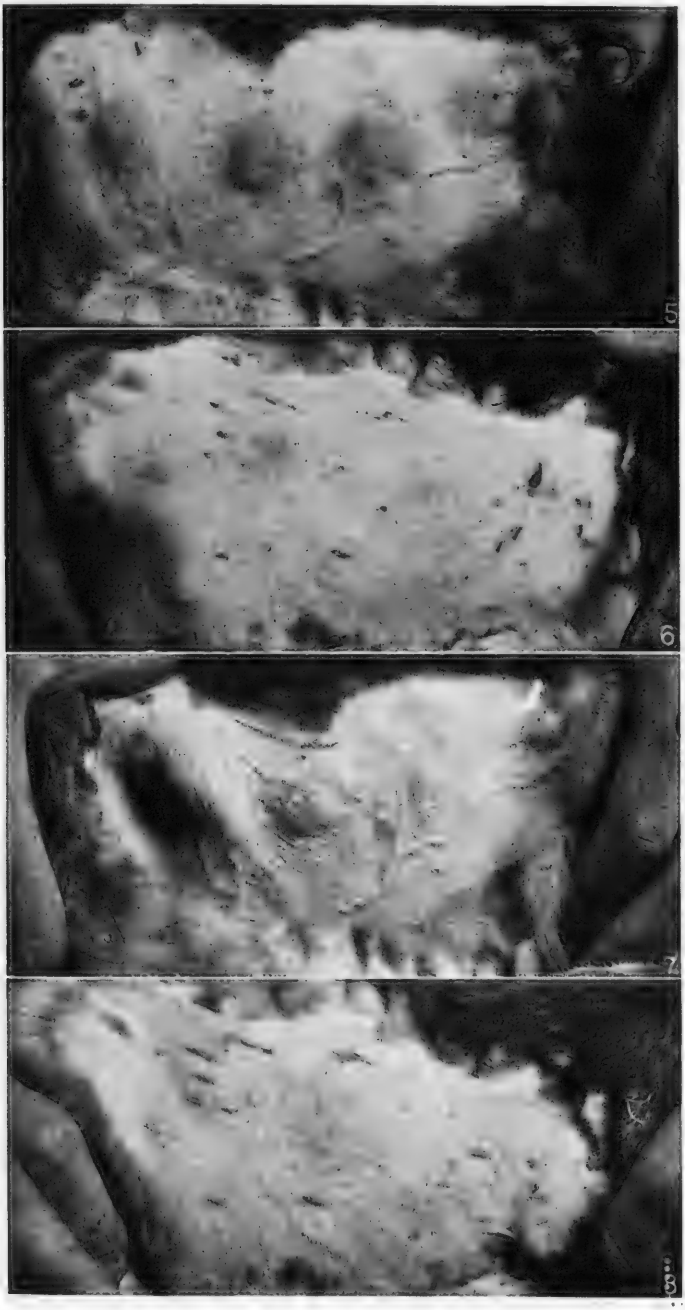
FIG. 6. Other side of rabbit of Fig. 5. Four areas of skin were infiltrated with 0.5 cc. of immune serum. 24 hours later these areas were inoculated with portions of the same dilutions of the streptococcus suspension used on the side shown in Fig. 5. No lesions developed following the inoculations of streptococci into the skin prepared with immune serum. Photographed 2 days after the inoculations.

FIG. 7. Same as Fig. 5, 5 days later.

FIG. 8. Same as Fig. 6, 5 days later.



(Rivers and Tillett: Local passive immunity.)



(Rivers and Tillett: Local passive immunity.)

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

VI. The Relative Susceptibility of Different Strains of Mice to per Os Infection with the Type II Bacillus of Mouse Typhoid (Bacillus pestis caviæ).

By IDA W. PRITCHETT, Sc.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 31, 1924.)

During the past year, as part of the studies on experimental epidemiology being carried on at the Rockefeller Institute, routine monthly tests have been performed in this laboratory with five separate strains of mice in an effort to determine as accurately as possible their relative resistance to infection, by the gastrointestinal route, with the Type II bacillus of mouse typhoid (*Bacillus pestis caviæ*). The histories of these strains, so far as they are known to us, are as follows:

Bagg Mice (*Albino*).—About six mice were obtained from the Carnegie Experiment Station at Cold Spring Harbor in December, 1922, and were bred at the Rockefeller Institute. They were received at Cold Spring Harbor in 1922 from Dr. L. C. Strong,[1] who had himself obtained them from Dr. H. J. Bagg in 1919. Dr. Bagg had first obtained these mice in 1913, and the strain as used by him arose from a single brother-sister mating. In our hands the strain has been strictly inbred, but not by brother-sister matings.

Hagedoorn Mice (*Albino*).—About ten mice were obtained from Dr. A. C. Hagedoorn[2] of the University of California in June, 1922, and were bred at the Rockefeller Institute. Dr. Hagedoorn obtained them in 1916 from Dr. T. B. Robertson,[3] who used them for experiments on growth. In Dr. Hagedoorn's

[1] Strong, L. C., *J. Exp. Zool.*, 1922, xxxvi, 67.

[2] Hagedoorn-LaBrand, A. C., and Hagedoorn, A. L., *Am. Naturalist*, 1920, liv, 368.

[3] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347. Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363.

hands they were inbred by brother-sister matings. In our hands, they have been strictly inbred, but not by brother-sister matings.

Lathrop Mice (*Black, with an Occasional Brown*).—About twenty-five mice were obtained from the Carnegie Experiment Station at Cold Spring Harbor in December, 1922, and were also bred at the Rockefeller Institute. This stock originally came to Cold Spring Harbor in 1920 from a breeder in Holyoke, Massachusetts, successor to Miss Lathrop. It was of heterogeneous origin, having arisen from a mixture of yellow, black, and brown mice. In our hands the strain has been constantly inbred, though not by brother-sister mating, and as the mice first received from Cold Spring Harbor were predominantly black, we have eliminated as far as possible, by selective breeding, the brown individuals, though these still arise in small numbers. Experiments on the effects of x-ray, performed with mice of this strain, have been described by Bagg and Little.[4]

Little Mice (*Dilute Brown*).—About six mice were obtained from the Carnegie Experiment Station at Cold Spring Harbor in December, 1922, and bred at the Institute. They were brought to Cold Spring Harbor in 1919 by Dr. C. C. Little of the University of Maine, who had inbred them since 1909 by brother-sister mating. In our hands they have been strictly inbred but not by brother-sister matings.

Rockefeller Institute Mice (*Albino*).—This stock originated at the Rockefeller Institute in 1917 from a combination of two albino strains, and in 1919 two new strains were added. Since that time our mice have been strictly inbred, without the addition of new stock, not, however, by brother-sister mating. They are the same mice that have been used in the work of Flexner,[5] Amoss,[6,8] and Webster,[7,9] on the epidemiology of mouse typhoid. Since our experience with these mice has been the most extensive, they have served as controls for the other strains in each experiment.

The importance of using large numbers, in order to diminish error from random sampling, has been kept constantly in mind, and whenever possible at least fifty mice of each strain were used in each of the monthly tests, a total of 3,120 mice having been employed in the course of the year. Intrastomachal inoculations were made according

[4] Little, C. C., and Bagg, H. J., *Am. J. Roentgenol.*, 1923, x, 975; Bagg, H. J., and Little, C. C., *Am. J. Anat.*, 1924, xxxiii, 119.

[5] Flexner, S., and Amoss, H. L., *Proc. Nat. Acad. Sc.*, 1921, vii, 319. Flexner, S., *J. Exp. Med.*, 1922, xxxvi, 9.

[6] Amoss, H. L., *J. Exp. Med.*, 1922, xxxvi, 25, 45.

[7] Webster, L. T., *J. Exp. Med.*, 1922, xxxvi, 71, 97.

[8] Amoss, H. L., and Haselbauer, P. P., *J. Exp. Med.*, 1922, xxxvi, 107.

[9] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 21, 33, 231, 269, 781; xxxviii, 33, 45; 1924, xxxix, 129, 879; *Am. J. Hyg.*, 1924, iv, 134.

to the technique described by Webster,[7] each mouse receiving 0.5 cc. of a 1:100 dilution made from an 18 hour broth culture of the mouse typhoid bacillus, the dose received by each animal containing on an average about 5,000,000 organisms.

EXPERIMENTAL.

The series of tests was begun in October, 1923. It was not possible at the outset to obtain the full number desired of all the five strains of mice, since some had bred too slowly, and in October and November one or more strains were completely lacking. By December, however, it was possible to obtain monthly a certain number of mice from each strain and thereafter the full quota of fifty from each strain was used almost every month. Occasionally this number was even exceeded.

The routine procedure in starting one of these tests was as follows:

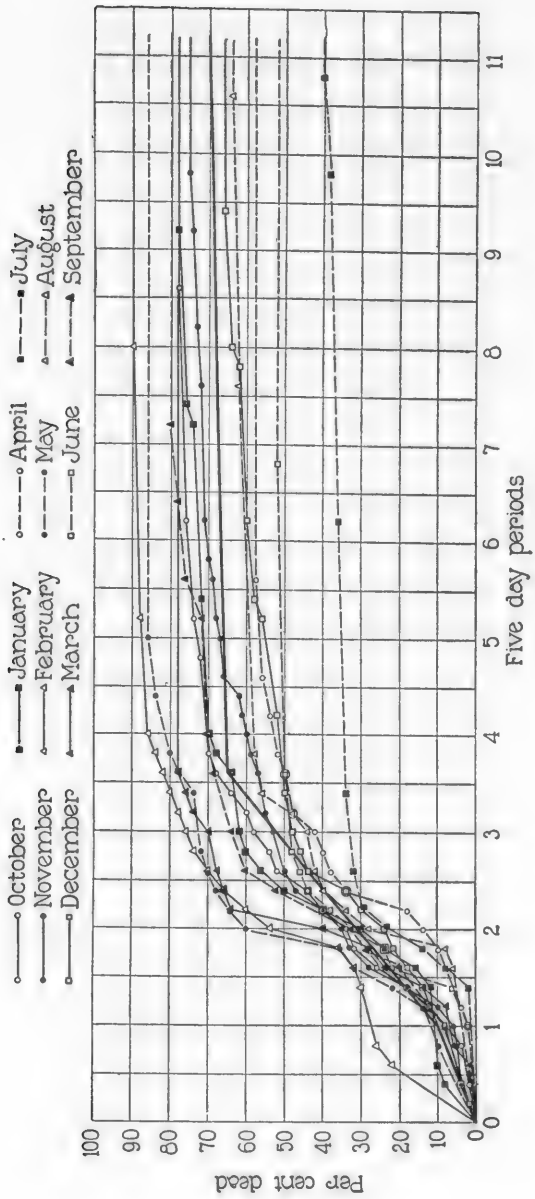
The mice from all strains were assembled, each mouse in a separate battery jar partly filled with shavings, on the day before inoculation. The next morning a dilution of 1:100 of an 18 hour broth culture of the mouse typhoid bacillus was made in bouillon from which three dilution plates were poured for the purpose of counting the organisms present. Each mouse then received by stomach tube 0.5 cc. of this 1:100 dilution of the original culture, containing usually about 5,000,000 mouse typhoid bacilli. All the animals were fed directly after the administration of the infecting dose, with the usual daily diet of bread soaked in pasteurized Grade B milk. At about 9 o'clock each morning the deaths for the preceding 24 hours were recorded. For the first 7 months, autopsies were performed on all dead mice and cultures made on green dye plates from the spleen and feces, to determine the presence of the mouse typhoid bacillus. However, after 1,256 autopsies had been performed, of which only 17 failed to reveal the presence of this bacillus in the spleen or feces or both, it was decided that the labor involved in performing the autopsies was scarcely justified in view of the small percentage of negative animals encountered, and they were consequently discontinued. All experiments were allowed to run 8 weeks.

A comparison of Text-figs. 1 to 6 will give a clear indication of the relative susceptibility of these five strains of mice to *per os* infection with the bacillus of mouse typhoid Type II (*Bacillus pestis caviæ*). Since the Rockefeller Institute mice, being the ones most intensively studied, have served as controls for the other four strains, we shall use them throughout as a common basis for comparison with these strains.

Three properties may be especially mentioned in connection with the curves of the Institute mice, (*a*) their height, (*b*) their pitch, and (*c*) their scatter. Discussion of the first and second properties will be deferred until after the question of scatter in the curves of all strains has been considered.

Text-fig. 1 shows the twelve monthly curves for the Institute mice, together with the standard control curve previously constructed for this strain by Webster.[10] The scatter in these curves is seen to be considerable, the highest and lowest curves being separated by a difference of 50 per cent at their termination. It must be noted, however, that this scatter, while marked, is not haphazard, the curves falling into three fairly clear-cut groups—high in the spring, low in the summer, and midway between the two extremes during the fall and winter. This phase of the work is more fully dealt with elsewhere.[11]

[10] Webster,[9] p. 231.
[11] Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 209.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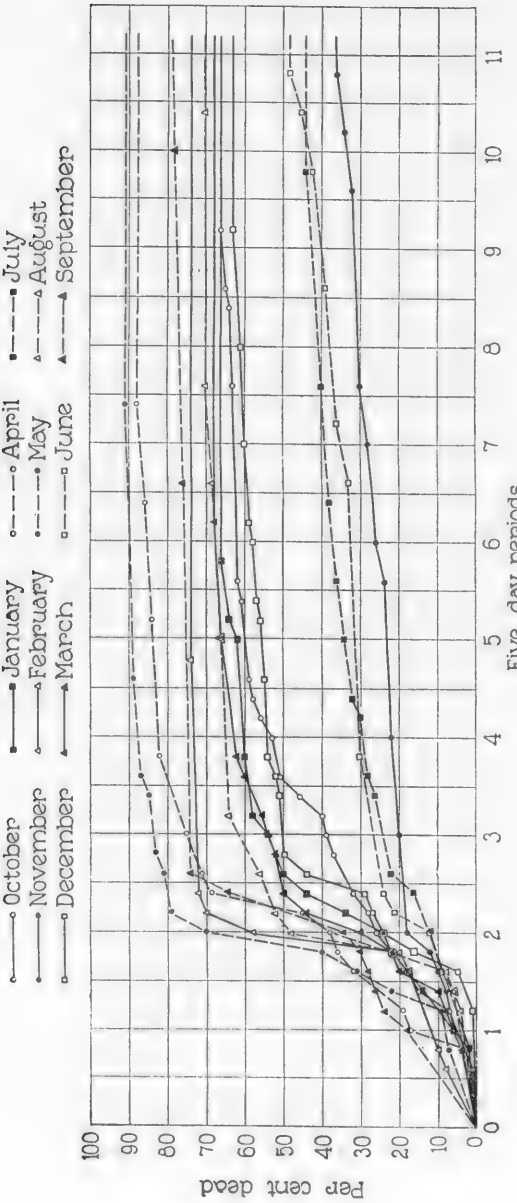
TEXT-FIG. 1.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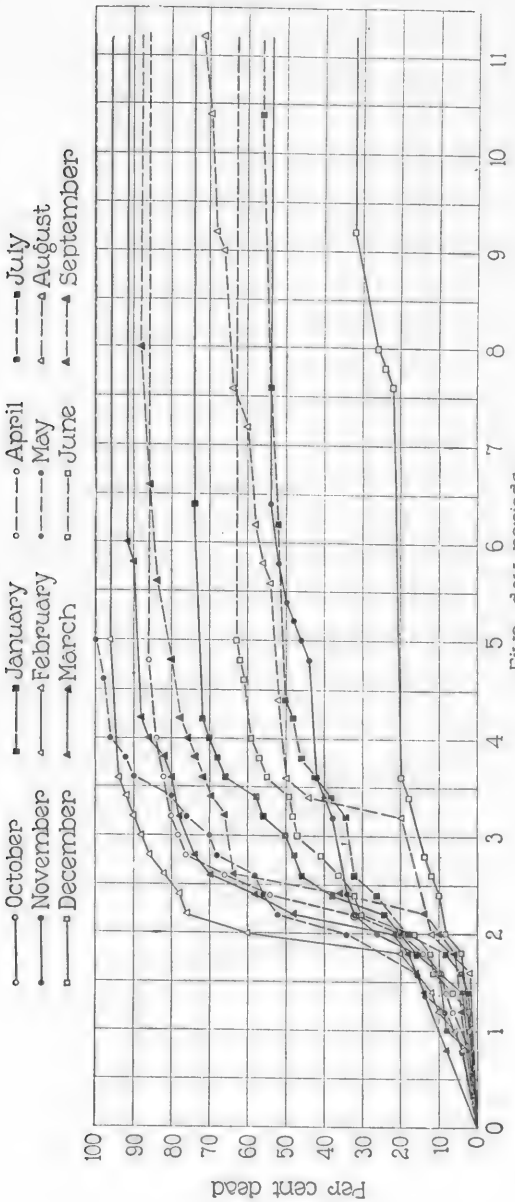
Five day periods

TEXT-FIG. 2.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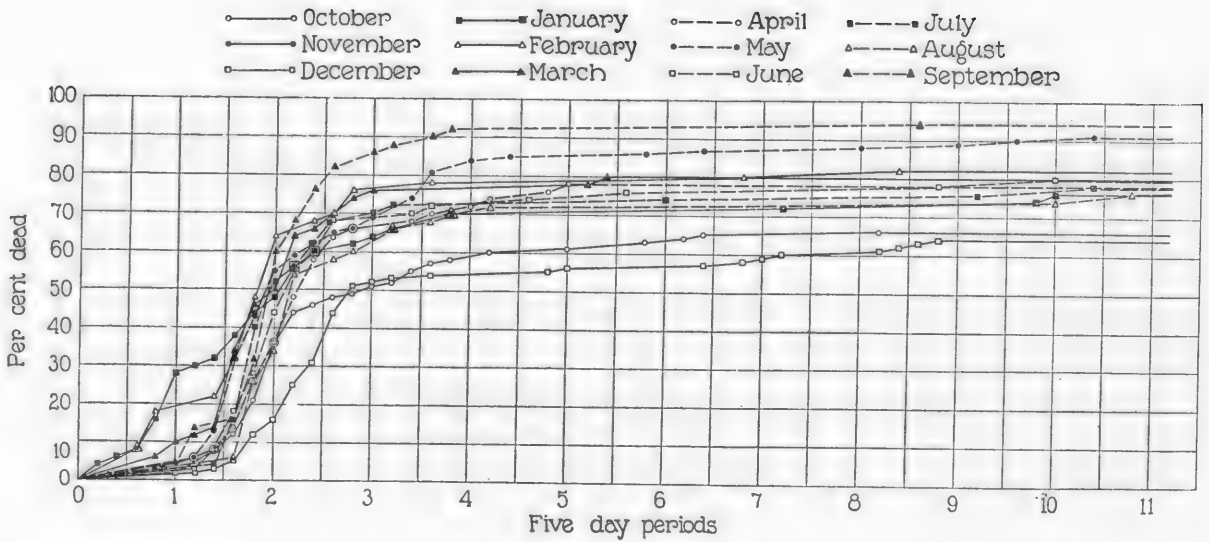
Five day periods

TEXT-FIG. 3.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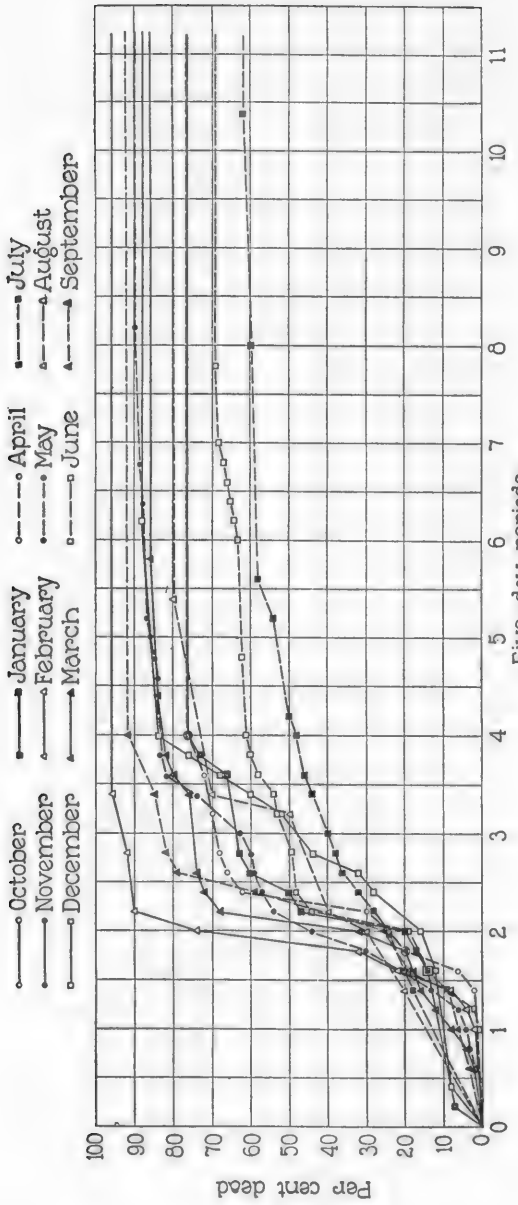
Five day periods

TEXT-FIG. 4.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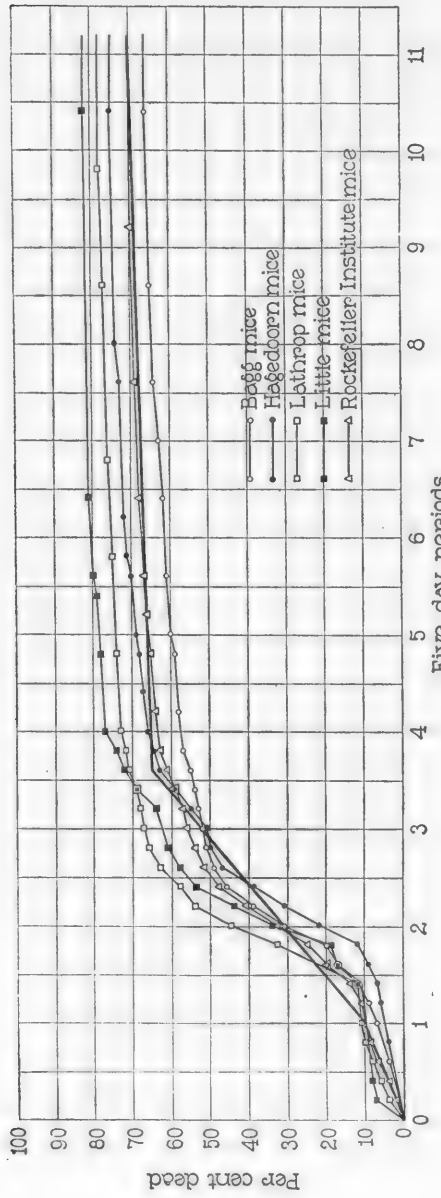
TEXT-FIG. 5.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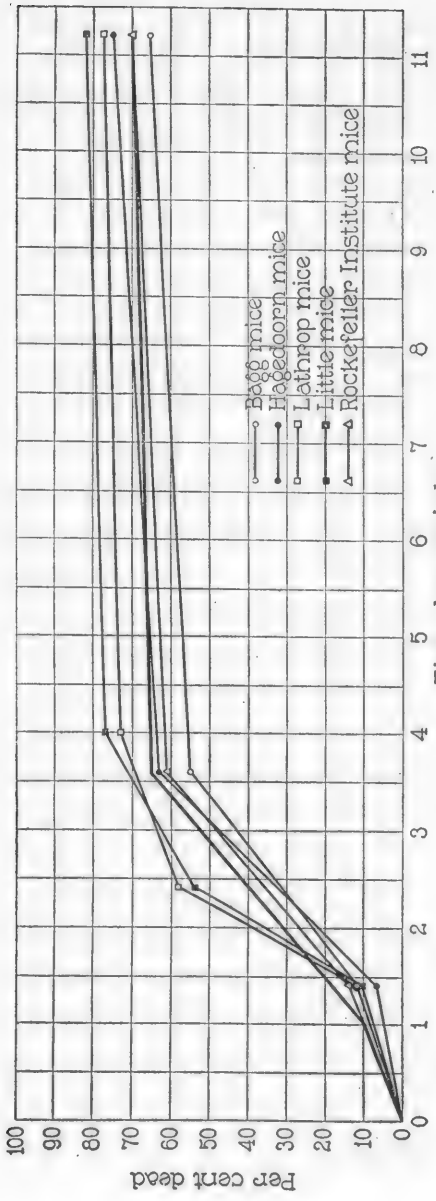
Five day periods
Bagg mice
Hagedoorn mice
Lathrop mice
Little mice
Rockefeller Institute mice

TEXT-FIG. 6.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Five day periods
Bagg mice
Hagedoorn mice
Lathrop mice
Little mice
Rockefeller Institute mice

TEXT-FIG. 7.

Text-figs. 2 to 5 show the monthly curves for the Bagg, Hagedoorn, Lathrop, and Little mice respectively. The scatter is seen to vary considerably between the different strains and between each strain and the Institute controls. If we take as an index of the scatter in the curves for each strain the difference between the lowest and the highest mortality recorded for that strain, the following figures are obtained (Table I).

Table I brings out the differences in the constancy of response of the different strains to the inoculation. As is shown also in Text-fig. 4, the Lathrop mice were the most constant in their response throughout the year, the lowest curve recorded being separated from the highest by a margin of only 30 per cent. The Hagedoorn mice, on the other hand, showed so wide a variation (68 per cent between the lowest

TABLE I.

Strain.	Difference separating lowest and highest mortality rates.
	per cent
Lathrop mice	30
Little "	34
Institute "	50
Bagg "	55
Hagedoorn "	68

and the highest curves) as to render somewhat unsatisfactory any determination of the average susceptibility of this strain to infection with the bacillus of mouse typhoid. This may be clearly seen from Text-fig. 3, in which are given the curves for the Hagedoorn strain during the whole year. The Institute and Bagg mice most closely resemble each other in the matter of the scattering of their curves as well as in the tendency of their curves to fall into three fairly clear-cut groups—high in spring, low in summer, and medium in fall and winter. The Little dilute brown mice incline rather toward the Lathrops in the constancy of their response to the inoculation.

The relative height and pitch of the curves for the different strains may best be studied in Text-fig. 6, in which are shown average curves constructed for each strain from all the curves for that strain in the course of the year, together with the standard control curve previously

constructed by Webster for the Institute mice. It will be seen from these curves that in general the pitch of the curves of the Bagg, Hagedoorn, and Institute mice is similar, while the curves of the Lathrop and Little mice are distinctly more abrupt in their rise. As to the relative susceptibility of the different strains, as shown by the final mortality figures from the average curves given in Text-fig. 6, the Littles are the least resistant, the Baggs the most resistant, while the Lathrop, Hagedoorn, and Rockefeller Institute mice fall in decreasing order of susceptibility between the two extremes.

In Text-fig. 7 are shown standard control curves constructed for each of the five strains of mice from the foregoing charts, together with Webster's standard control curve for the Institute mice.[10] These curves have been constructed to bring out especially the variation in the pitch of the curves for the several strains, as well as the difference in the final mortality rate of all the strains. It is interesting to see how closely the curve for the Institute mice in this test approximates that previously found by Webster for this strain.

Although the differences in the final mortality figures for the various strains of mice, as shown by the average curves, given in Text-fig. 6, are not great, we feel that they are significant since practically no deaths from intercurrent disease, with a consequent natural selection of the strongest, had occurred in any of the strains before inoculation and since the same relative differences in susceptibility to mouse typhoid in general predominated in the separate monthly experiments.

DISCUSSION.

The only recent work known to us which seems to parallel our own closely is that of Wright and Lewis[12] on tuberculosis in the guinea pig. These authors worked with several distinct families of guinea pigs inbred through many generations, and were able to distinguish clear-cut differences in the relative resistance of these families to tuberculous infection. Moreover, they showed that individuals of both sexes in the most resistant family were capable of transmitting the factor for resistance to their offspring, the progeny from such a mating prov-

[12] Wright, S., and Lewis, P. A., *Am. Naturalist*, 1921, lv, 20.

ing to be superior in resistance to the more resistant parent. Variations in the susceptibility of different strains of mice to transplantable mouse tumors have been observed by many authors, most recently perhaps by Strong,[13] who records for a great variety of strains of mice receiving transplants of a mouse sarcoma that there was practically 100 per cent susceptibility in normal individuals, except in the case of wild mice, in which there was approximately 80 per cent susceptibility. Webster[10] has recorded dissimilarity in the susceptibility of three strains of mice to infection with the bacillus of mouse typhoid used in this laboratory. Twenty-six mice from a Pennsylvania breeder and twenty-eight mice from a New Jersey breeder, together with twenty control mice of the Rockefeller Institute strain, were inoculated *per os* in the usual way with a suspension of M. T. II. The resulting mortality curves showed that the New Jersey mice resembled the Institute mice in their susceptibility to the infection, while the Pennsylvania mice were far more resistant.

The interest in these observations lies not alone in the mere demonstration that variations in susceptibility to injurious agents do exist among different strains of the same species, receiving the same food and subjected to the same living conditions, but in the bearing that such an observation must have on the correlation of experimental work done in any given biological field with totally unrelated strains of the same species of animal. It emphasizes anew the importance of knowing thoroughly the normal range of variation in susceptibility for any given strain of experimental animal, before attempting to draw a sharp line between the normal and the abnormal response to an injurious agent.

SUMMARY.

Five separate strains of mice have been tested for their relative susceptibility to *per os* infection with the Type II bacillus of mouse typhoid (*Bacillus pestis caviæ*), more than 500 individuals of each strain having been employed in the course of 12 months. Clear-cut differences in the susceptibility of these strains to the infection have been shown to exist.

[13] Strong, L. C., *J. Exp. Med.*, 1924, xxxix, 447.

MICROBIC VIRULENCE AND HOST SUSCEPTIBILITY IN PARATYPHOID-ENTERITIDIS INFECTION OF WHITE MICE.

VII. Seasonal Variation in the Susceptibility of Different Strains of Mice to per Os Infection with the Type II Bacillus of Mouse Typhoid (Bacillus pestis caviæ).

By IDA W. PRITCHETT, Sc. D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 31, 1924.)

In another paper[1] brief mention has been made of the fact that seasonal variations in the susceptibility of mice to *per os* inoculation with the bacillus of mouse typhoid have been observed. In the present communication we wish to deal more fully with this observation, and by a rearrangement of the mortality curves to emphasize the point more strongly.

The mice employed in this study are the ones previously referred to.[1] They consisted of 3,120 animals, arising from five separate strains which have been described in some detail in the previous paper. It was not possible in October and November to obtain the desired number of mice of all strains, and in these 2 months, therefore, one or more strains were completely lacking. The total number of mice employed in each strain was as follows: Bagg mice (albino), 685; Hagedoorn mice (albino), 580; Lathrop mice (black), 675; Little mice (dilute brown), 530; Rockefeller Institute mice (albino), 650. Total, 3,120.

EXPERIMENTAL.

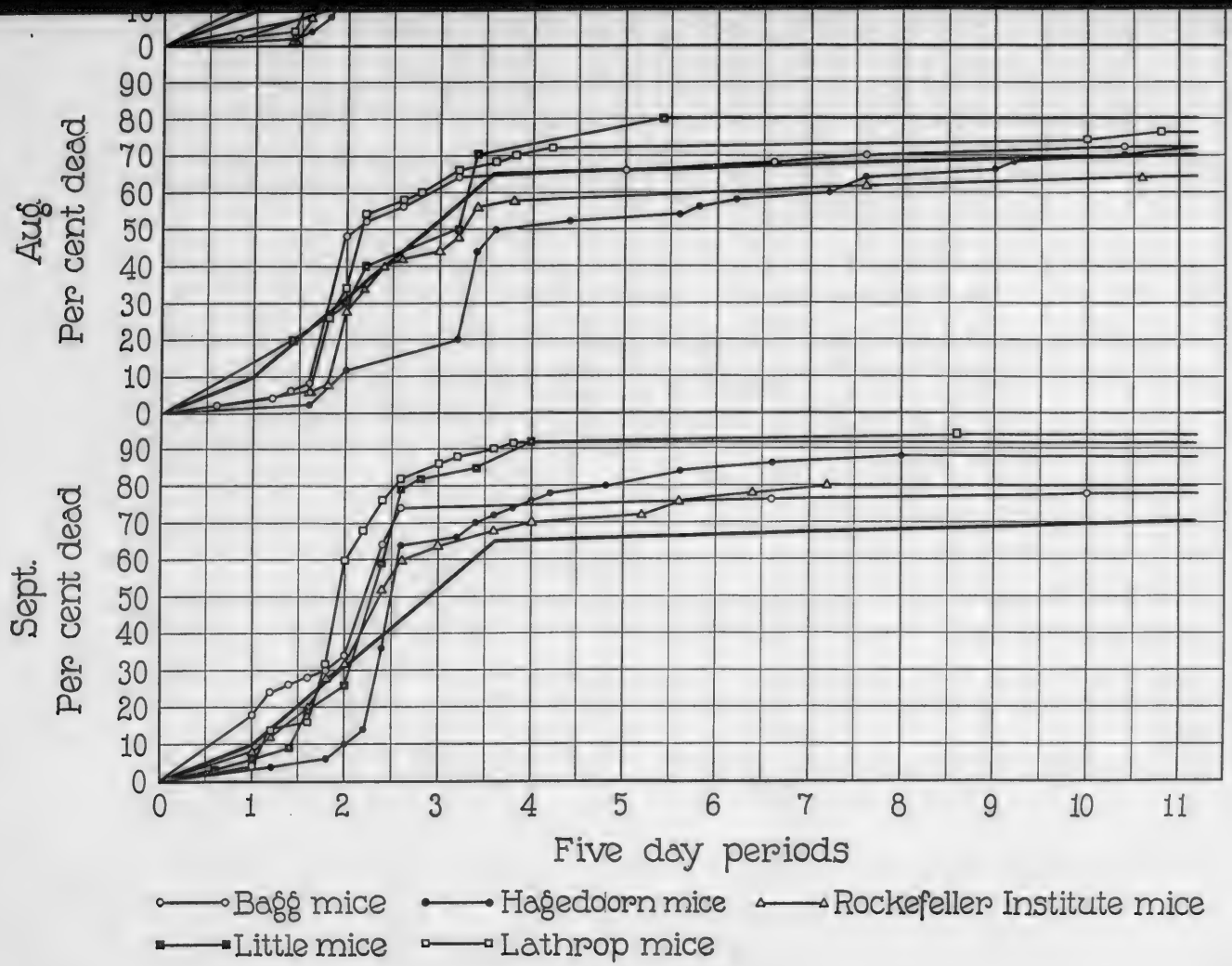
As noted in the preceding paper, routine monthly tests were made with these five strains of mice throughout a year. On the day before they were inoculated with the mouse typhoid bacillus each mouse was placed in a separate battery

[1] Pritchett, I. W., *J. Exp. Med.*, 1925, xli, 195.

jar partly filled with shavings. The next morning a dilution of 1:100 of an 18 hour broth culture of M. T. II was made in bouillon, from which three dilution plates were poured for the purpose of counting the organisms present. Each mouse then received by stomach tube 0.5 cc. of this 1:100 dilution of the original culture, containing on an average about 5,000,000 mouse typhoid bacilli. The whole series of mice was fed directly after the inoculation with the usual daily diet of bread soaked in pasteurized Grade B milk. Each experiment was allowed to run for 8 weeks, during which time the deaths were recorded daily and mortality curves plotted.

The results of the monthly tests are shown in Text-fig. 1. From above downward the charts are arranged in chronological order, and in each chart is included, in addition to the curves for the five strains of mice, Webster's[2] standard control curve for the Rockefeller Institute strain. This curve, remaining constant throughout all the charts, serves as a graphic indicator of the changes from month to month in the height and pitch of other curves. It is evident that a seasonal rhythm has occurred in all these strains of mice to inoculation with the bacillus of mouse typhoid. As has been briefly noted in the preceding paper, the curves tend to fall, with some irregularities, into three classes: (*a*) the high spring curves, (*b*) the low summer curves, and (*c*) the fall and winter curves which roughly represent the average between the two extremes. These three classes of curves are most sharply indicated by the Bagg and Rockefeller Institute mice. The Hagedoorns clearly show the marked spring rise (February to May inclusive), followed by a drop in mortality during June and July to a level comparable to that of the curves of the preceding fall and winter; but the summer mortality, while lower than in the spring, is not the lowest for the year, so that a high spring group of curves and a lower group composed of summer, fall, and winter curves are all that can be distinguished. The case of the Little mice is somewhat similar. Unfortunately the October and November curves had to be omitted for this strain because of an insufficient supply of mice at that time, but from December to May inclusive, and again in August and September, practically all the curves are high and steep, while the low group of curves is represented by those for June and July, and the median group is altogether lacking.

[2] Webster, L. T., *J. Exp. Med.*, 1923, xxxvii, 231.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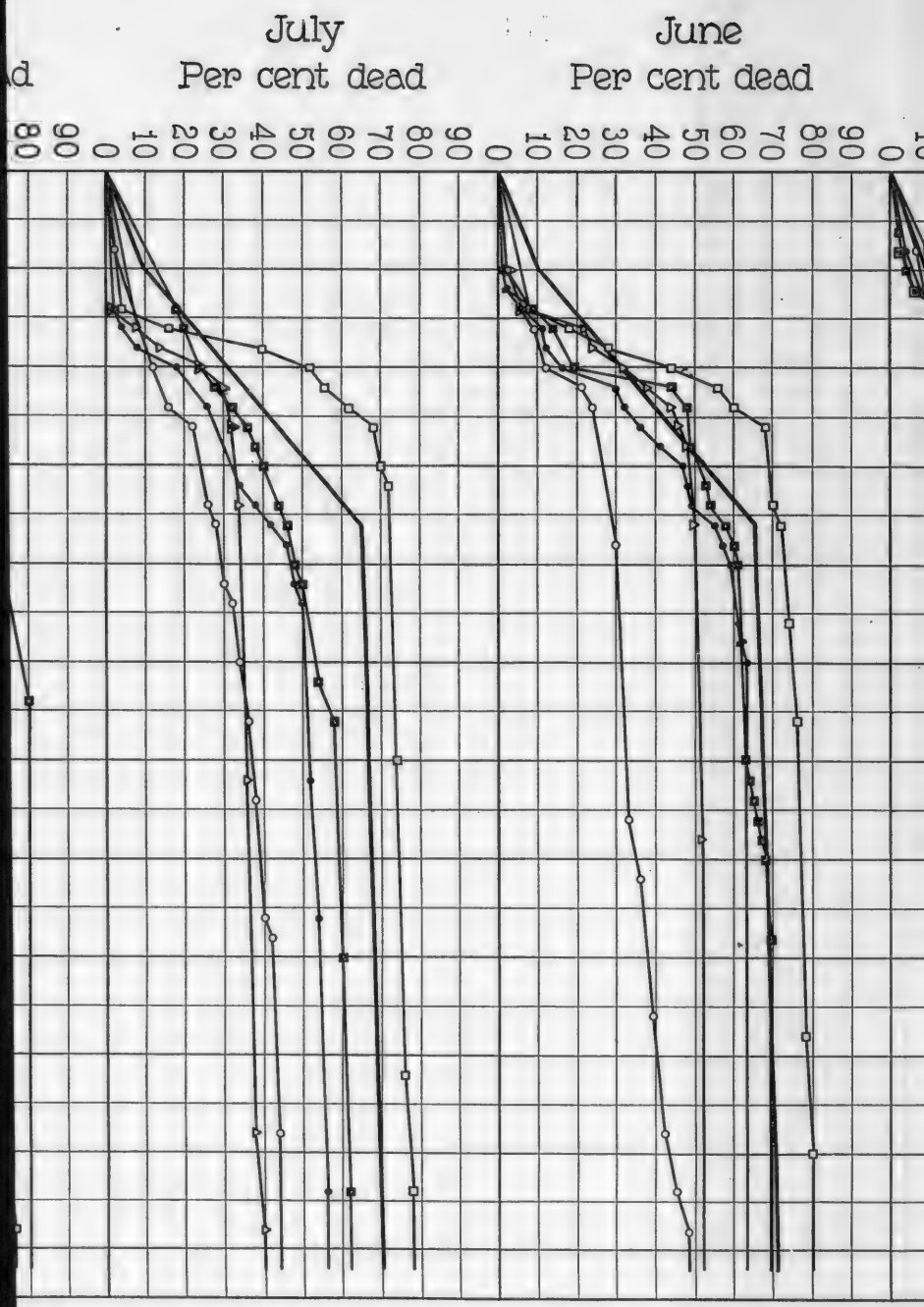
Five day periods
Bagg mice
Hagedoorn mice
Rockefeller Institute mice
Little mice
Lathrop mice

TEXT-FIG. 1.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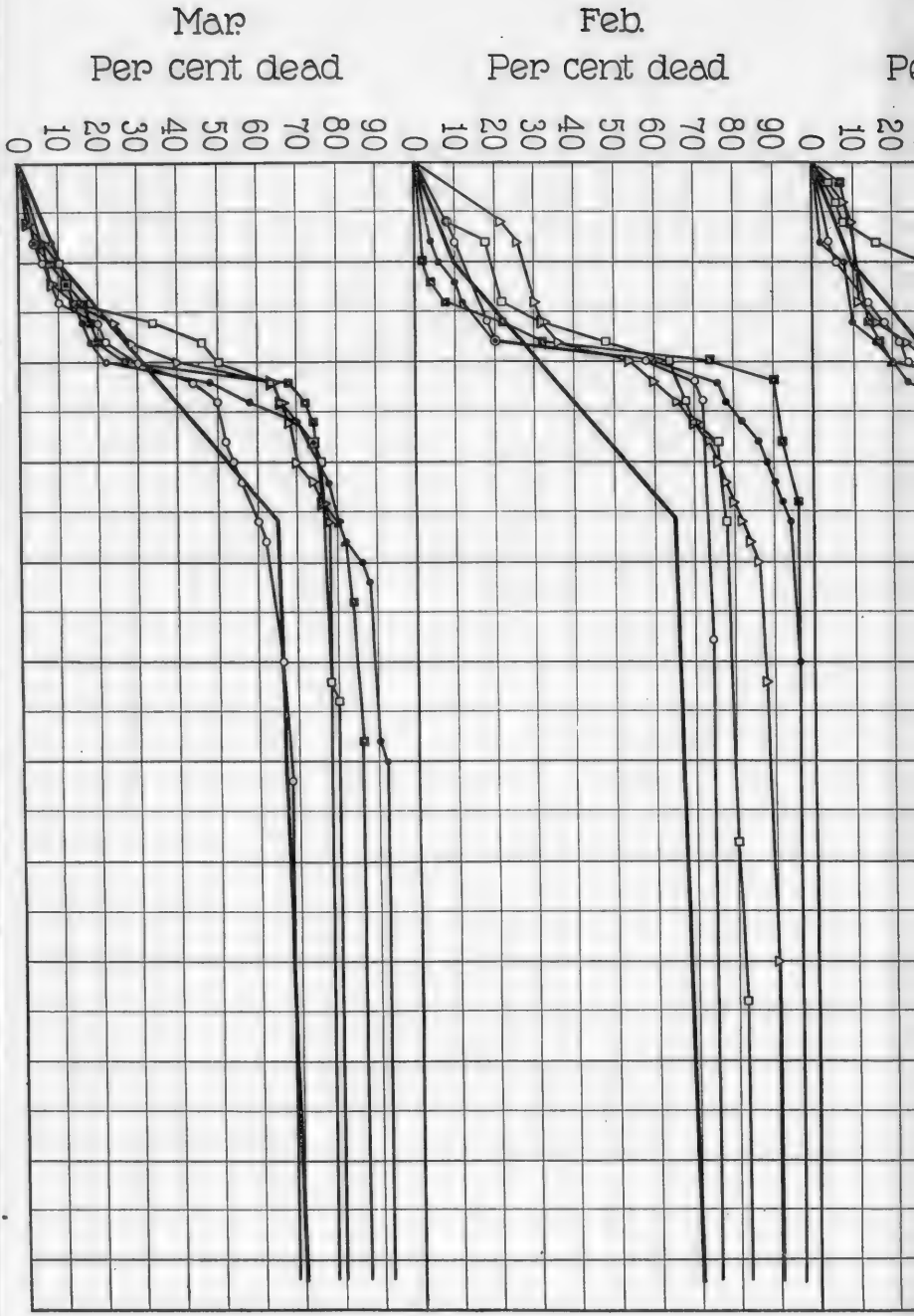
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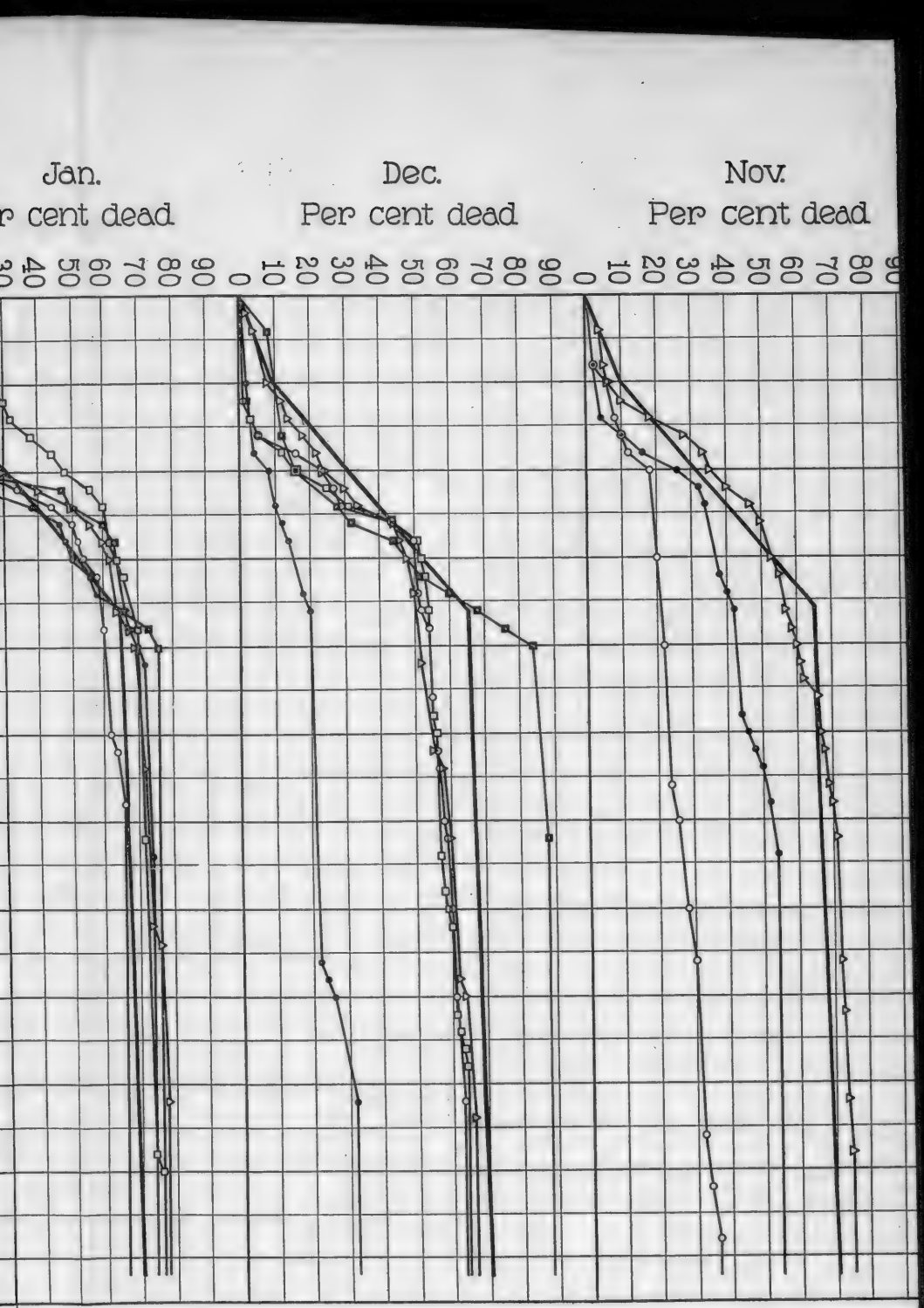
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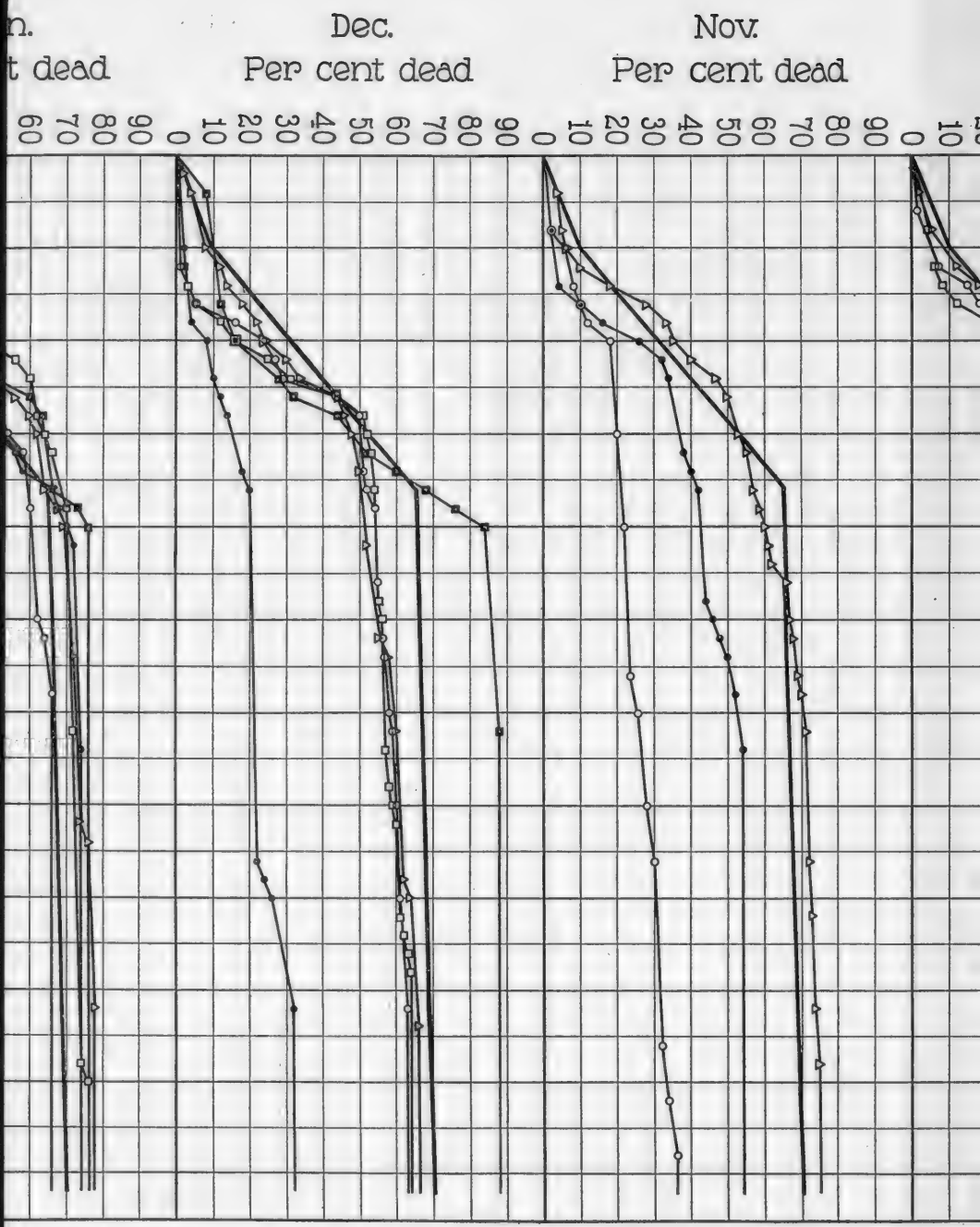
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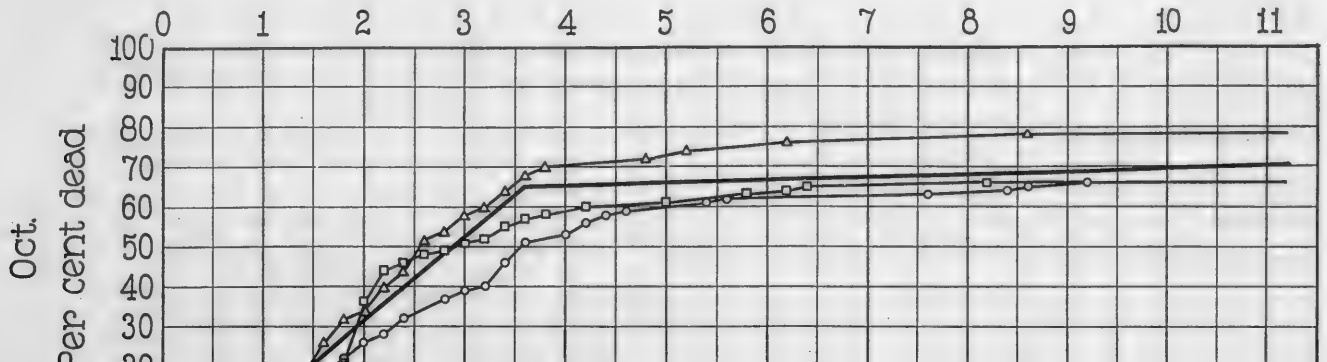
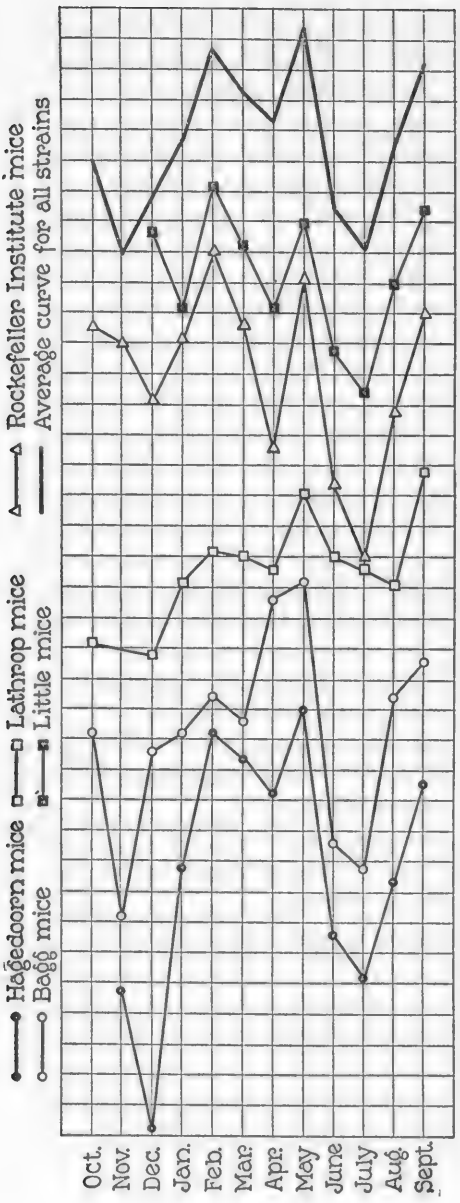
0 10


0
1
2
3
4
5
6
7
8
9
10
11
100
90
80
70
60
50
40
30
Per cent dead
Oct.


Hagedoorn mice
Bagg mice
Lathrop mice
Little mice
Rockefeller Institute mice
Average curve for all strains
Oct.
Nov.
Dec.
Jan.
Feb.
Mar.
Apr.
May
June
July
Aug.
Sept.

TEXT-FIG. 2.

It should be noted that although the Lathrop mice differ from the other strains in that they show very little variation in their response to the inoculation throughout the year, such seasonal fluctuations in susceptibility as they exhibit correspond closely to the more marked fluctuations occurring in the other four strains. This may be clearly seen from a study of Text-fig. 2, which shows curves constructed for each strain from the total mortality figures obtained in the successive monthly experiments, together with an average curve for all strains. The months are indicated on the abscissa, and the curves are placed one above the other at such short intervals that, while no two curves overlap, the form of all curves can be readily compared. In general it may be seen that there are two spring peaks in the curve of each strain, one in February and one in May. In the curves of the Bagg, Hagedoorn, and Lathrop strains, as well as in the average curve for all strains, the May peak is the higher, while in the Little and Rockefeller Institute strains the February peak surpasses the May peak. Nevertheless in general the form of all curves is the same, such differences as exist being, for the most part, quantitative. In all strains there is a high mortality level during the spring, a lower level during the summer, and in the fall a sharp increase in mortality rate.

DISCUSSION.

Seasonal variation in the susceptibility of rabbits to a transplantable tumor of rabbit origin has recently been described by Brown, Pearce, and Van Allen.[3] In a second paper[4] they record observations on seasonal fluctuation in the weight of various rabbit organs and call attention to the correlation observed between both tumor susceptibility and organ weight and the available hours of sunlight. Even more recently a paper has appeared by Elton[5] on periodic fluctuations in the death rate among normal animals of various species in which the influence of climatic changes is also stressed.

[3] Brown, W. H., Pearce, L., and Van Allen, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 371.

[4] Brown, W. H., Pearce, L., and Van Allen, C. M., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 373.

[5] Elton, C. S., *Brit. J. Exp. Biol.*, 1924, ii, 119.

For the most part the recent papers on seasonal variations in susceptibility concern themselves primarily with diet deficiencies.

Since Webster[6] has shown that the virulence factor for paratyphoid-enteritidis strains is relatively constant and since, under the conditions of our tests, the dosage factor has been rigidly controlled, we feel justified in assuming that the seasonal variations in mortality rate recorded for these mice are due to fluctuations in host susceptibility. The strain of M. T. II (*Bacillus pestis caviæ*) used in the tests is kept on agar at a constant temperature (+4°C) throughout the year, and transferred to fresh agar at regular intervals. It has had no animal passage for more than 2 years. Nevertheless when transferred to broth and inoculated in doses containing approximately equal numbers of bacilli it has given rise to a distinct seasonal rhythm in the mortality rate of the five strains of mice employed.

That the seasonal variation in mortality rate observed in these tests will be duplicated next year with the mouse typhoid bacillus or with another organism cannot be foretold. Indeed, we have formed the impression that the accentuation of spring curves noted may be unusual, and that the seasonal fluctuations here recorded may be more pronounced than would often be the case. It is already apparent that the curves for September are far higher than those obtained in the preceding autumn months, which may presage an even higher mortality average for all strains this year than last. Though we have not heretofore made any exact study of the occurrence of seasonal variation in the susceptibility of our mice, it has been commonly observed in this laboratory since the early days of the work on the epidemiology of mouse typhoid that the mortality curves of mice taken from the regular Institute stock tend to be lower during the summer months than those of similar mice inoculated during the autumn and winter. It was for this reason that Webster, in constructing his standard control curve for the Institute mice, used only the curves of mice inoculated during the winter months, believing them to be more representative than the lower summer curves. A solid experimental basis for this belief is provided by the 12 curves constructed during the past year for the Institute mice,

[6] Webster, L. T., *J. Exp. Med.*, 1923, xxxviii, 45.

of which the average for the autumn and winter closely approximates Webster's standard control curve and lies about midway between the high spring and the low summer curves.

SUMMARY.

Seasonal variations are described in the response of five strains of mice to inoculation with the Type II bacillus of mouse typhoid (*Bacillus pestis caviæ*). There occurred in the case of all strains a high peak of mortality during the spring, a lower death rate during the summer, and a subsequent autumn rise in the number of deaths.

CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

I. An Exotic Strain of Encephalitogenic Virus.

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INTRODUCTION.

The experimental study of encephalitis has entered upon a new phase. Opened to exploration by the investigations of Strauss[1] and his coworkers on epidemic encephalitis and by Grüter[2] and Löwenstein[3] on experimental febrile herpes, the generalizations adopted by them or their immediate successors are now in process of revision. In view of the seriousness of the situation arising out of the epidemic invasions of encephalitis between 1917 and almost the present date, it was perhaps to have been expected that, given any degree of success attending the experimental studies on encephalitis, they should have led to premature conclusions.

It is only recently that the results of these studies have come to be considered critically. Hence many of the earlier deductions have been found excessive and in need of rectification. The sources of some of the main errors have become evident from a more precise examination of the experimental material—the rabbit chiefly—employed.[4] Another error, which perhaps should have been more effectually guarded against, arose from lack of due consideration being accorded the evidences which suffice to establish relationship of cause and effect between a given microorganism and a recognized pathological process or disease.

[1] Strauss, I., Hirshfeld, S., and Loewe, L., *New York Med. J.*, 1919, cix, 772.

[2] Grüter, W., *Klin. Monatsbl. Augenheilk.*, 1920, lxv, 398.

[3] Löwenstein, A., *Münch. med. Woch.*, 1919, lxvi, 769; *Klin. Monatsbl. Augenheilk.*, 1920, lxiv, 15; 1920, lxv, 399.

[4] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

The founders of bacteriology were confronted with this problem and, of course, with the pitfalls inseparable from this class of experiments. Out of the safeguards erected by them there emerged the so called postulates of Koch which, until recently, dominated the conceptions of bacteriologists working in the etiological field. These postulates were, moreover, formulated at a period in which bacteriologists were dealing with microscopically visible organisms which also could be secured in artificial cultures. If, therefore, these elaborate precautions were found necessary then, how much more so, it may be inquired, are they demanded now that we are seeking to connect the far more subtle and elusive, invisible, so called filterable, parasites with particular kinds of specific disease. Our experimental methods should, under these circumstances, have become more rigorous, which has not, in fact, always been the case.

Hitherto there have been described two varieties or classes of encephalitis-inducing virus: one supposedly obtained from cases of epidemic encephalitis,[1,5] the other definitely obtained from the vesicular contents of febrile herpetic eruptions.[6] As these two kinds of virus have been more and more studied, they have been shown to possess common properties and to be indistinguishable by any biological tests now available.

The so called virus of encephalitis has been secured under circumstances not always free from suspicion of doubt. Of the, on the whole, small number of specimens of this virus described, the larger part has been derived from inoculations either of nasopharyngeal secretions or of brain substance taken at autopsy. The former material, even when filtered, cannot be regarded as a simple inoculum, and the latter has oftener than not been taken from organs removed in the usual course at autopsy and thus subject to gross contamination.

The case is different with the reported successful inoculations of the cerebrospinal fluid withdrawn during life from cases of encephalitis in man. This fluid is readily obtained free of extraneous microorganisms. There can be no doubt that in certain instances the

[5] Levaditi, C., and Harvier, P., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 354; *Ann. Inst. Pasteur*, 1920, xxxiv, 911.

[6] Grüter, Löwenstein, and many others.

injection of cerebrospinal fluid into the brain of rabbits sets up an encephalitis which is transmissible in indefinite series from rabbit to rabbit. In this instance everything depends on the interpretations put upon the results achieved and whether they are held to be due to the action of the true virus of encephalitis or some other agent capable of inducing an inflammation of the brain in the rabbit.

The experiments to be reported in this paper derive their particular value from the significant fact that a virus, on the one hand indistiguishable from the so called virus of encephalitis, and on the other hand, from the virus of febrile herpes, has been obtained from the cerebrospinal fluid of a patient suffering from vascular and neural syphilis, who had been under continuous observation for more than 2 years, and who never presented symptoms of epidemic encephalitis.[4]

History of the Case Yielding the Exotic Virus.

The patient, J. B., from whom the virus was obtained was a male, 51 years old, treated at the Presbyterian Hospital, New York. We are indebted to the physicians of the hospital for the cerebrospinal fluid of this and of many other cases, employed in our experiments. The patient had been ambulatory since February, 1921. He attended the dispensary, would enter the hospital for intraspinal, salvarsan medication, and then return to his work. The Wassermann reaction was several times found positive in the blood and cerebrospinal fluid. The patient was still under observation in September, 1923. At no time in this long period had symptoms other than those referable to the syphilitic infection been complained of or observed.

Scope of the Investigation.

The experimental reproduction in animals of a condition resembling epidemic encephalitis has engaged our attention from 1919 onward. When the first fatalities caused by the disease occurred in New York, we secured, through the kind cooperation of the pathologists of several hospitals, portions of the brain or the entire organ as removed at autopsy for the purpose of inoculating animals. In view of the successful use of *Macacus rhesus* in the experimental study of poliomyelitis, our first inoculations were made on this species. No unequivocal successful result was secured. Following the announcement of the successful use of rabbits by Strauss and his coworkers, this species was employed by us but again without success.

The method we pursued was to select material from the basal ganglia in as clean a manner as possible. When the whole or half the brain came into our possession it was wrapped in sterile towels and transported at once to the laboratory. As a rule the exposed outer portions of the brain could be sliced away, exposing the ganglia, the chief seat of the inflammatory lesions, which often proved sterile on culture tests. The fragments were emulsified and injected subdurally or intracerebrally into the anesthetized animals, and portions were, at the same time, placed in glycerol and also in fixing fluids, the last for histological preparations.

As Strauss and his coworkers continued to report successful inoculations of rabbits, especially with filtered washings of the nasopharynx and of filtered extracts of nasopharyngeal mucosæ, removed at autopsy, and finally with cerebrospinal fluid, our efforts to secure transmission of a virus were made to include all those materials but again without a single unequivocal result. The confirmatory reports of the work of Strauss by Levaditi, and then by Doerr[7] led us to extend our search much more widely, for which purpose we employed the cerebrospinal fluid from a large number of clinical cases diagnosed as epidemic encephalitis as well as other cases of varied nervous and other affections. For the opportunity to carry out this wider study we are indebted to the clinical staffs of the Presbyterian Hospital and the Neurological Institute. In the course of this comprehensive study one of us (H.L.A.) became connected with the Johns Hopkins Hospital, Baltimore, where he carried out similar inoculations. Finally, an outbreak of epidemic encephalitis arose at Winnipeg, Canada, and Dr. A. J. Douglas courteously sent specimens of cerebrospinal fluid, enclosed in thermos bottles, from eight cases. Except the last specimens, which were put into rabbits about 12 days after the collection, all the fluids were injected within 24 hours of their withdrawal.

Procedures.

Two methods of injection were followed in this inclusive investigation. At least two anesthetized rabbits received the injection of 0.35 cc. of each cerebrospinal fluid into the forebrain through a trephined opening, and at least two others received 1 cc. intracranially by simple lateral needle puncture through the suture of the skull behind and above the level of the outer canthus of the left eye in the manner employed by Strauss, Hirshfeld, and Loewe.[1] Very few rabbits were lost from pressure. The fluids were always tested for sterility in deep bouillon tubes and for globulin by the Noguchi method. A given specimen of the cerebrospinal fluid was injected into from two to five rabbits. For the miscellaneous fluids two rabbits were used;

[7] Doerr, R., and Schnabel, A., *Schweiz. med. Woch.*, 1921, ii, 469; *Z. Hyg. u. Infectionskrankh.*, 1921, xciv, 29.

for the fluids coming from supposed cases of epidemic encephalitis, five or more were injected. The inoculated rabbits were kept under observation for many weeks. They were individually inspected twice a day. Any animal showing symptoms, no matter how trifling, was closely watched: the rectal temperature was taken, it was tested for impaired movement, excitation, salivation, etc.

The miscellaneous collection of cerebrospinal fluids included cases of syphilis (many), brain tumor (several), general nervous affections, including multiple sclerosis (a number), acute infections—pneumonia, epidemic meningitis, influenza, etc. (a number).

The number of deaths in the large series of rabbits inoculated was small. In a single instance only was a disease encountered which was identified with the condition described in the literature as "experimental epidemic encephalitis." As will appear presently, our view is that the disease does not experimentally reproduce epidemic (lethargic) encephalitis, but that it is induced by a living agent or virus and hence we have preferred to designate the condition "virus encephalitis." Our belief is also that this virus encephalitis is indistinguishable from the experimental form of encephalitis induced in the rabbit by the virus of febrile herpes, with which it may be and probably is, identical. Aside from this one instance of fatal virus encephalitis, all the fatalities among the inoculated rabbits arose from injury (fighting), snuffles, or other secondary diseases. All the rabbits *in extremis* were etherized and submitted to complete autopsy and bacteriological examination. Particular attention was directed to the central nervous organs, which were also submitted to histological study. In all doubtful instances reinoculation of brain material into other rabbits was made immediately in order to guard against the loss of any virus strain possibly present, and because, as is well known, the first implantation of virus into the rabbit is difficult, while the subsequent transfers are readily effected.

The total number of specimens of cerebrospinal fluid injected into rabbits was 100, of which 27 were derived from patients diagnosed or suspected as being cases of epidemic encephalitis, and 73 from cases of miscellaneous character.

EXPERIMENTAL RESULTS.

On Jan. 4, 1923, two rabbits were given 0.35 cc. of cerebrospinal fluid, from patient J. B., by the intracranial route. One of the rabbits failed to show symptoms. On Jan. 13 the other was noted to be tremulous, to turn in circles to the left (side of inoculation), to move the head rhythmically backwards and forwards, and to grind the teeth (gnashing). The rectal temperature was 105.6°F. There was no salivation. Death occurred the next day. The autopsy showed congestion of the brain and meninges and normal-appearing other organs. Three rabbits were injected intracranially on Jan. 14 with brain suspension of this animal. Other portions of the brain were preserved in 50 per cent glycerol and taken for histological examination.

A second specimen of the cerebrospinal fluid of J. B. was injected into five rabbits, by the intracranial method, on Feb. 2 and a third specimen into four rabbits (two into the brain, two into the eye after scarification of the cornea) on Sept. 20, but without results.

The strain of virus from the rabbit succumbing on Jan. 14 was transferred to other rabbits, in indefinite series, with ease and in them produced regularly the train of symptoms and the pathological effects to which the name of experimental encephalitis is applied. This strain of virus has been employed in a comparative study of the effects of the so called virus of epidemic encephalitis and the virus contained in febrile herpes vesicles, and it has been found indistinguishable from them by any of the known biological tests.

Series A. Intracranial Inoculation.

On Jan. 14 three rabbits were injected intracranially with a 10 per cent suspension of the brain (virus) of the rabbit succumbing to the inoculation of the cerebrospinal fluid of patient J. B.

Rabbit A received 0.25 cc. of the suspension. Jan. 16. The animal was slow, showed a rectal temperature of 106.5°F., gnashing of teeth, salivation, tremor, and later convulsions. Jan. 17. Etherized. The brain was congested; urinary bladder distended. Ordinary cultures showed no bacteria.

Rabbits B and C were given respectively 0.3 and 0.35 cc. of the same brain suspension. 24 hours later there were noted fever, rapid respiration, spasmodic contractions of the abdominal muscles, attended by cough, then prostration, convulsions, and urine retention. One died and the other was etherized when moribund on Jan. 17. The brain was sterile for ordinary bacteria.

The results of the inoculation of the above three rabbits show the J. B. virus to possess high activity or virulence, and to be capable of inducing the train of clinical events to which the term experimental virus encephalitis is applicable. A long series of experimental inoculations, extending over nearly 3 years, has been carried on with

the J. B. virus and in that time no falling off of potency has been noted. On the other hand, the virus has shown itself glycerol-resistant, active by means of paper and Berkefeld filtration, and by other modes of inoculation than the intracerebral. Some protocols of animals injected with paper and Berkefeld filtrates follow. In respect to glycerolation, it may be stated that samples of the brain which were kept at 4°C. in glycerol for more than 6 months showed undiminished activity.

May 21, 1923. Two rabbits received 0.05 and 0.025 cc. of a paper filtrate of a 5 per cent emulsion of fresh virus (brain). Both animals developed characteristic symptoms, including fever, salivation, muscular agitation, convulsions, and urine retention. Death occurred on the 7th and 8th days respectively.

Oct. 28. Two rabbits were given 0.35 cc. of a Berkefeld filtrate prepared from a 10 per cent suspension of brain (virus), glycerolated for 6 weeks. Typical symptoms appeared on the 4th, and death followed on the 9th and 10th days respectively.

Series B. Corneal Inoculation.

That the brain tissue taken from rabbits succumbing to inoculation with the so called encephalitis virus is capable of inducing a kind of herpetic keratitis in the rabbit was pointed out by Levaditi and Harvier[8] after the demonstration of the similar effect of the herpes virus on the cornea of rabbits by Blanc and Caminopetros.[9] That the J. B. virus can excite a corresponding inflammation of the cornea is shown by the following protocols.

On Jan. 14, 1923, the cocainized eye of Rabbit G was incised with a cataract knife previously dipped into a suspension of the brain of the original rabbit succumbing to the J. B. virus. 48 hours to 4 days later there were noted marked suppuration and keratoconjunctivitis. The local effects progressed and became very severe, and on Jan. 22 were attended by general symptoms, consisting of tremor, gnashing, falling to the side of the inoculated eye (left), and fever (106°F.). Death took place on the 9th day.

The brain from this animal was used for intracranial inoculation of another rabbit which developed, within 48 hours, high temperature and paralysis of the extremities. On the 5th day convulsions and death occurred. From this animal Rabbit H was inoculated into the cornea. Within 72 hours a severe kerato-

[8] Levaditi, C., and Harvier, P., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 300.
[9] Blanc, G., and Caminopetros, J., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 629.

conjunctivitis occurred, followed on the 7th day by tremor, head turning, salivation, urine retention, convulsions, and death.

On Sept. 9, 1924, two rabbits were given intracorneal inoculations of J. B. virus taken from a fresh brain which came from the animal succumbing to injection of the 6 months glycerolated specimen of virus. Both rabbits developed severe keratoconjunctivitis and typical symptoms of encephalitis and succumbed on the 8th and 9th days respectively.

These protocols could be considerably increased in number. They suffice, however, to show that the J. B. virus possesses definite and pronounced affinity for the rabbit cornea in which it multiplies, provoking a severe keratoconjunctivitis, whence it regularly invades the brain, setting up a fatal encephalitis. The virus is transmissible in series from cornea to cornea, from cornea to brain, and from brain to cornea.

In the course of a long series of inoculations into the cornea, the fact was noted that glycerolation tends to diminish numerically the viable microorganisms constituting the virus. That is, if the long glycerolated virus is implanted directly upon the cornea, a milder keratoconjunctivitis results, from which recovery may occur, attended by subsequent immunity; while if the same glycerolated specimen is introduced into the brain, a fatal encephalitis results. The cornea inoculated from the brain of such an animal invariably develops severe inflammation which, in turn, leads to encephalitis and death. These facts indicate not a mitigation of virulence through the action of the glycerol but a partial destruction of the organisms within the brain tissue. And in conformity with this effect experiments could be cited in which an occasional specimen conserved in glycerol has lost entirely its power to infect rabbits, even when injected directly into the brain.

Series C. Skin Inoculation.

Just as the so called virus of encephalitis has been shown capable of inducing keratitis in the rabbit, Levaditi, Harvier, and Nicolau[10] have shown it to possess, in common with the virus of febrile herpes, power to excite a vesicular dermatitis in this animal. As the fol-

[10] Levaditi, C., Harvier, P., and Nicolau, S., *Compt. rend. Soc. biol.*, 1921, lxxxv, 287.

lowing examples attest, the J. B. virus acts on the skin of rabbits in an identical manner.

On Jan. 23, 1924, Rabbit I was shaved over the left side of the abdomen and the skin abraded with two scratches 1 inch long. The area was then smeared with fresh brain from a third transfer of J. B. virus. Suppuration and vesiculation, most marked along the scratches, followed on the 6th day. General symptoms appeared and death took place on the 11th day. A second rabbit (J), treated in the same manner, reacted in the same general way. On the 16th day tremor and paralysis of the extremities were notable; and death occurred on the 17th day.

With the brain tissue of Rabbit I, Rabbit K was given a skin inoculation which was followed by vesicular dermatitis, ataxia, urine retention, and death on the 10th day.

J. B. virus attacks the skin surface as it does the corneal tissue, and, as in the case of the latter, tends invariably to pass from the skin into the interior of the nervous system and to excite a fatal encephalitis. The glycerolated J. B. virus acts on the skin in a manner in all essential respects identical with the fresh (brain) virus.

Series D. Blood Inoculation.

The herpes and so called encephalitis viruses are believed to pass by way of the peripheral nerves to the central nervous organs. This view has been especially developed by Goodpasture and Teague[11] and employed by them to explain the head and body turnings. The same general considerations apply to the J. B. virus. Moreover, it has been found that when the herpes and encephalitis strains of virus are injected into the blood encephalitis also follows. An identical effect is produced by the intravenous inoculation of the J. B. virus.

On May 10, 1924, Rabbits L and M received respectively an intravenous injection of 2 and 3 cc. of a suspension of fresh brain tissue taken from a rabbit developing encephalitis 5 days after an intracerebral inoculation of J. B. virus. Both developed general symptoms consisting of fever (106°F.), tremor, gnashing, salivation, muscular excitement, including rhythmical erection of the body, and retention of urine. Death resulted on the 17th day.

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[11] Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923–24, xliv, 139.

Series E. Nasal Inoculation.

The power to penetrate external surfaces, including the nasal mucous membrane, is possessed by the J. B. virus in common with the other varieties of virus referred to.

On Jan. 23, 1924, there was inserted into the left naris of Rabbit N a cotton plug carrying fresh brain pulp taken from a rabbit succumbing to J. B. virus encephalitis. This animal developed the usual train of general symptoms, including fever, tremor, circling movements (to the left), and urine retention. Death occurred on the 7th day.

Series F. Testicular Inoculation.

The discovery by Noguchi that the testicle is a favorable site of multiplication of vaccine virus led to the employment of the testicle for the cultivation of other kinds of virus. Levaditi[12] showed that this organ suffices also for the class of viruses we are dealing with and that from the testicle an invasion of the brain took place.

On May 13, 1924, Rabbit O received an intratesticular injection of 1 cc. of a 10 per cent suspension of fresh (brain) J. B. virus. May 23. The organ was swollen and edematous. 0.3 cc. of exudate was aspirated and injected into the testicle of Rabbit P. In addition to the inflammation of the testicles, both animals developed general symptoms, including fever, salivation, tremor, palsy, and convulsions, and died on the 10th and 17th days respectively. The virus was transmitted further from these two animals both by testicular and by intracranial inoculation.

The intratesticular mode of inoculation differs in one respect from the other, perhaps more direct means of inoculation of the virus employed, in that remission of symptoms may be observed. After a first period of fever there is a return to normal, and then after a further interval of several days, a return of the fever, the appearance of general symptoms, and the occurrence of death.

Histology of Virus Encephalitis.

In keeping with the local effects following the direct inoculation of the virus into the eye, skin, and other parts, histological lesions,

[12] Levaditi, C., Ectodermoses neurotropes. Poliomyélite, encéphalite, herpès, Monographies de l'Institut Pasteur, Paris, 1922, 132, 138.

more or less profound, are encountered. These have been described repeatedly for the herpes and encephalitis viruses; they do not differ in respect to the J. B. virus. In this section, therefore, attention will be centered on the microscopical changes met with in the nervous system, the brain chiefly, of the inoculated rabbit. It would naturally be supposed that with the symptoms of inflammation of the brain so severe and striking there would appear correspondingly profound lesions in that organ. As a matter of fact, what is found is a wide disparity of lesions and no close relationship between their intensity and the severity of the symptoms.

The mode of inoculation affects the character of the lesions, which tend to be more marked when the intracranial injection of virus has been made. In this instance several factors cooperate: trauma, positively chemotactic tissue elements, and virus. Usually, therefore, a meningoencephalitis results, in which the polymorphonuclear leucocyte takes a considerable part. The injection of a Berkefeld filtrate instead of a tissue suspension diminishes but does not abolish the local reaction. To determine, therefore, the effect of the virus itself, it is necessary to study the brain of rabbits into which the virus has penetrated from the skin, eye, blood, etc.

It might be expected that the examination of a suitable series of brains from this class of animals would yield quickly a definitive result. Such is, however, by no means the case; at least it has not been the case with our animals. It is true that the local traumatic and other effects present in intracerebrally inoculated rabbits are wanting. But the lesions in general met with are subject to wide fluctuations of situation and severity. This disparity has proved puzzling in the past and has led certain experimenters into error and the making of false inferences.

The difficulty arises, in part, from the nature of the experimental animal—the domestic rabbit—itself. At least this is true of the American laboratory rabbit, and certain evidences point to the same being true for the rabbits employed by continental investigators. The apparently healthy domestic rabbit exhibits not infrequently a variety of encephalitis—inflammation of the brain—in the preinoculation condition, which leads to a degree of confusion in interpreting

the actual virus lesions, often not to be resolved.[13] The circumstance that wide and profound indications of encephalitis are often found in the rabbit in which no recognizable symptoms have appeared, is notable because, when the virus form of encephalitis arises, it is attended by symptoms of great severity and gravity. Hence it would seem that the inflammatory lesions alone cannot be held to account for the symptoms, from which it follows that beside provoking perivascular and interstitial mononuclear cell invasions the virus attacks nerve cells directly, either stimulating them to overfunction, or paralyzing their activity altogether. The clinical symptoms observed in virus encephalitis clearly show these two opposite effects.

With the stocks of rabbits employed by us, it has often not proven possible to tell when one variety (old) and the other variety (new) of lesions began and ended. The old variety of lesions, it has now been shown, is induced in part by a microsporidial parasite, *Encephalitozoon cuniculi* (Levaditi),[14] first observed in 1922 by Wright and Craighead,[15] and rediscovered recently by Levaditi and Nicolau. Since, in the meantime, this parasite has been shown to localize in the kidney, in which it regularly induces inflammation, and since it is only accidentally and incidentally deposited in the brain, where its growth excites a varying degree of cellular reaction, it is of dubious propriety to emphasize in its name the encephalitogenic property.[13] Among the preinoculation brain lesions are many in which no parasites of any kind have been detected; they are regarded as scars or residues, perhaps, of a microsporidial infection and possibly of still other kinds of infection. It is this class of lesions from which the main difficulty arises in determining the effects to be attributed, on the one hand, to the virus and, on the other hand, to preexisting infection.[16]

However, so far as this discussion concerns the manner of action of the J. B. virus on the brain of rabbits, it may be stated that no distinction can be made between the effects of this virus, the herpes

[13] Smith, T., and Florence, L., *J. Exp. Med.*, 1925, xli, 25.

[14] Levaditi, C., Nicolau, S., and Schoen, R., *Compt. rend. Acad.*, 1923, clxxvii, 985.

[15] Wright, J. H., and Craighead, E. M., *J. Exp. Med.*, 1922, xxxvi, 135.

[16] McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.

virus, and the so called encephalitis virus. And this is true, not only as regards the histological changes themselves, but also as respects the minute, chromatic, intracellular bodies described by Lipschütz, Da Fano, and others.[17] There is little probability that these bodies are microorganisms; they are more probably degeneration products. It is of interest to record that Parker[18] failed to find corresponding bodies in the human brain from cases of epidemic encephalitis.

Although the mouse[19] occasionally shows the microsporidial parasite in the brain, the guinea pig and white rat are free from a similar infestation. The three species are, however, subject to virus encephalitis, and respond, on intracerebral inoculation, with profound inflammatory lesions and severe symptoms. Unfortunately these animals do not respond with encephalitis to virus inoculation elsewhere in the body than the brain; hence the traumatic and virus effects are not readily disentangled.

Hitherto, what may be spoken of as the direct action of the virus on the nerve cells of the brain has been considered little, if at all. Attention has been directed especially to the perivascular and other infiltrative lesions. But since, as we have seen, this latter class of lesions may prevail on a large scale without provoking symptoms, it is probable that in virus encephalitis another kind of action, involving more directly the nerve cells themselves, comes into play. Since the symptoms of virus encephalitis provide indications of the parts of the brain especially affected, the minute study of nerve cells in the affected regions is something open to exploitation, and is, indeed, in progress.

[17] Lipschütz, B., *Arch. Dermatol. u. Syph., Orig.*, 1921, cxxxvi, 428. Da Fano, C., *Brit. Med. J.*, 1921, i, 153; 1921, ii, 652; *J. Path. and Bact.*, 1923, xxvi, 85. Levaditi, C., Ectodermoses neurotropes. Poliomyélite, encéphalite, herpès, Monographies de l'Institut Pasteur, Paris, 1922, 164. Goodpasture, E. W., and Teague, O., *Proc. Soc. Exp. Biol. and Med.*, 1922–23, xx, 400. Cowdry, E. V., and Nicholson, F. M., *J. Exp. Med.*, 1923, xxxviii, 695.

[18] Parker, F., Jr., *J. Med. Research*, 1923–24, xliv, 289.

[19] Cowdry, E. V., and Nicholson, F. M., *J. Am. Med. Assn.*, 1924, lxxxii, 545; *J. Exp. Med.*, 1924, xl, 51.

Immunity Reactions.

It is admitted that the virus of herpes and the so called virus of encephalitis produce in the rabbit identical immunity effects. When an animal infected with these viruses recovers it becomes, after the lapse of a few weeks, insusceptible to reinoculation in any manner to either of the two varieties of virus. In process of identification of the J. B. strain of virus suitable tests were carried out to determine the immunological reactions it would display.

J. B. vs. J. B. virus.—Mar. 20, 1923. Rabbits 1 and 2 were inoculated upon the scarified skin and scarified cornea respectively with glycerolated J. B. virus. The former showed no effect; the latter developed keratoconjunctivitis. The inoculations were repeated on Apr. 24 with no effect in either animal. On May 29 both animals received an intracranial injection of 0.1 cc. of a paper filtrate of a 5 per cent suspension of fresh (brain) J. B. virus. Rabbit 1 succumbed typically to virus encephalitis; Rabbit 2 proved immune.

It is obvious that the glycerolated J. B. virus used was too weak to induce infection in Rabbit 1, which therefore remained susceptible, while Rabbit 2, which had recovered from a mild keratoconjunctivitis, was protected against the intracerebral inoculation of the active virus.

Through the kindness of Dr. Levaditi we came into possession of a specimen of his encephalitis virus, variety (*souche*) C. The next protocol records a cross-test with the J. B. and Levaditi strains of virus.

J. B. vs. Levaditi Virus.—Jan. 2, 1923. Rabbits 3 and 4 received skin inoculations of Levaditi virus. Mild inflammation, vesiculation, and desquamation followed in both. On Mar. 29, Rabbit 3 received an intracerebral injection and Rabbit 4 a corneal inoculation of J. B. virus. The control animal developed typical virus encephalitis; neither of the others showed any effects whatever.

J. B. vs. Herpes Virus.—Feb. 26, 1923. Rabbit 5 was given a skin inoculation with herpes virus (strain Stewart). Inflammation with desquamation, followed by recovery, ensued. Apr. 28. Rabbit 6 had left naris plugged with cotton carrying Stewart herpes virus. Mild general symptoms with recovery resulted. On Sept. 18 both rabbits and a control received a corneal inoculation of J. B. virus. The control succumbed characteristically to virus encephalitis, while the other rabbits were unaffected.

The preceding set of tests shows conclusively that the J. B. virus is immunologically indistinguishable from a strain of so called encephalitis virus and a strain of herpes virus. In this respect, as in all other respects, as experimentally determined, the J. B. virus corresponds with the other varieties of encephalitogenic virus.

Action on Other Rodents.

The usual strains of herpes and encephalitis viruses are effective when injected into the brain of the guinea pig, mouse, and rat, and they are ineffective in the pigeon and usually in the monkey. We have injected the J. B. virus into the brain of pigeons, young dogs, and *Macacus rhesus* monkeys without success. The guinea pig, mouse, and rat, when so inoculated, respond with severe and fatal encephalitis. The brain of these animals shows, on microscopical study, marked, acute, widespread inflammation.

Guinea Pigs.—Two animals were given intracerebrally 0.1 cc. of a 10 per cent suspension of J. B. virus (rabbit brain). 4 days later both were tremulous, ataxic, gnashing, and salivating, and one turned in circles to the inoculated side (left). Convulsions set in and death occurred on the 5th and 6th days respectively.

Rats.—Two white rats were injected on the same day and in the same manner. They developed typical symptoms of encephalitis and died on the 4th day.

Mice.—Two white mice received each 10 drops of the same suspension of virus. Both developed the typical symptoms and died on the 3rd day. One mouse showed remarkable rhythmic, rapidly circling movements, followed by convulsions, prostration, and death.

SUMMARY AND CONCLUSIONS.

In this paper is given an account of an inoculable virus disease produced in the rabbit with cerebrospinal fluid taken from a case of vascular and neural syphilis.

The study which yielded the results presented was undertaken in the course of an investigation into the etiology of epidemic or lethargic encephalitis.

Twenty-seven samples of cerebrospinal fluid, derived from cases of epidemic encephalitis, were tested by us upon rabbits without positive result. The one successful instance in which an inoculable disease was produced arose from the injection of one of three speci-

mens of the cerebrospinal fluid taken from the case of syphilis. Following this success, two subsequent injections of the fluid, taken from the same patient, were made unsuccessfully.

Although certain American and European investigators have reported securing a virus from the cerebrospinal fluid of cases of epidemic encephalitis, we have consistently failed in our endeavors to confirm their results. However, we believe that the finding of the J. B. virus may serve to clarify the obscurity and confusion now enveloping the so called virus of encephalitis.

It had previously been shown that no biological differences could be detected between the herpes and the encephalitis strains of virus. The former, as is well known, is readily secured by inoculating rabbits with the contents of herpes vesicles, while the latter has, at best, been obtained with great difficulty.

The J. B. virus agrees biologically with the herpes and encephalitis strains of virus. It is our opinion that the J. B. virus is merely a herpes virus which has gained access to the cerebrospinal fluid and, at the time of inoculation of the rabbits, was present in a concentration sufficing to induce virus encephalitis.

The fact, if fact it is proved to be, that the herpes virus may find its way into the cerebrospinal fluid opens to question all the supposed instances of successful implantation of a virus of epidemic encephalitis upon the rabbit. It is indeed highly probable that, in so far as such a virus has been found at all in the cerebrospinal fluid, it also is a specimen of the herpes virus.

Our studies lead us to suppose that at best it is an infrequent event for the herpes virus to occur in demonstrable form in the cerebrospinal fluid. Perhaps a more delicate means of detection than the rabbit inoculation would serve to reveal the presence oftener. It is known that strains of herpes virus of greater or less intensity of action for rabbits exist. It is, of course, possible that we discover, by present methods, only the highly active strains and those only when chancing to be present in a certain concentration. We inoculated 100 specimens of cerebrospinal fluid and obtained in a single instance the virus infection of the rabbit.

In all respects the J. B. virus agrees in intensity of effect, in mode of attack upon the cornea, skin, and brain, and in immunization re-

sponses, with the true strains of herpes virus and the so called strains of encephalitis virus.

If, as the above statements indicate, all the virus strains of the class considered are examples of the herpes virus, it follows that the etiology of epidemic encephalitis remains entirely unresolved. It is highly improbable that the ubiquitous herpes virus plays the kind of part in human pathology which it has been shown to play in experimental rabbit pathology. While the active strains of that virus possess a strong affinity for the brain structures of the rabbit, the virus has not in the past shown any selective affinity for the brain of man. To ascribe epidemic encephalitis in man to particular and peculiar varieties of the herpes virus is, with our present knowledge, unwarranted.[20]

The wide variations in histological lesions described in the brain of rabbits succumbing to herpes or virus encephalitis raise the question of the essential manner of action of the virus upon the brain tissues. Hitherto it has been the cellular infiltrative lesions which have been emphasized. We have, however, learned that very extensive infiltrations about blood vessels and in the brain substance may exist independently of even mild symptoms of disease.[16] The question is propounded, therefore, whether the herpes virus does not attack nerve cells directly, affecting them quantitatively in such ways as at one time to produce stimulation and at another time paralysis. The manifold symptoms of virus encephalitis in the rabbit are open to this interpretation. In order, however, to base this notion on microscopical findings, a more subtle technique than hitherto widely employed is required. A histological restudy of the subject is being made with this view in mind.

The name virus encephalitis is proposed for the experimental disease produced in rabbits by the inoculation of the herpes and allied viruses.

[20] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1924, xc, 1372. Levaditi, C., Nicolau, S., and Poincloux, P., *Compt. rend. Soc. biol.*, 1924, xc, 1376.

CONTRIBUTIONS TO THE PATHOLOGY OF EXPERIMENTAL VIRUS ENCEPHALITIS.

II. Herpetic Strains of Encephalitogenic Virus.

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In the course of the investigation of the etiology of epidemic encephalitis, the question of different varieties or strains of the encephalitogenic virus has often arisen.[1] The practice, up to a short time ago, has been to separate the so called encephalitis virus from the virus of febrile herpes and, in spite of their marked similarities, to regard them as fundamentally, or, as respects their etiological activities, distinct. It is true that this rule has been somewhat relaxed recently, but the essential question whether, within the two main classes of virus, substrains or varieties are pathogenetically distinguishable has remained an open one. And yet upon the answer returned to this question rests, in large measure, the pending decision as to complete or only partial identification of the supposed virus of encephalitis with the proved virus of herpes.

In two recent papers Levaditi[2] has reported upon experiments which have led him and his coworkers to conclude that the two classes of virus compose a group for which they propose the designation "herpetico-encephalitic." According to their conception, there may be distinguished within the group encephalitogenic as distinct from the ectodermic varieties of such a virus, the former showing a particular power of attaching itself to neural (brain) structures. It is, indeed, this especial neurotropic kind of the herpetico-encephalitogenic virus which constitutes the provocative virus of epidemic encephalitis. But, of itself, even this variety is impotent to excite encephalitis

[1] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

[2] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1924, xc, 1372. Levaditi, C., Nicolau, S., and Poincloux, P., *Compt. rend. Soc. biol.*, 1924, xc, 1376.

in man, for which there is required, in addition, a peculiar susceptibility on the part of the nervous organs which only exceptional persons (or monkeys) display. In other words, in order that an epidemic of encephalitis may arise, there must coincide a mass modification of the soil (nervous system) and prevalence of a specially adapted variety of virus (herpetico encephalitic). The conjunction of these two factors in many parts of the world in the last half dozen or more years is made responsible for the many outbreaks of epidemic encephalitis reported.

According to the point of view just outlined, certain virus strains derived from cases of encephalitis in man are to be regarded as possessing a special affinity for the central nervous organs. One such strain is *souche* (variety) C of Dr. Levaditi. Thanks to his courtesy, we possess this strain and have, therefore, been able to compare its mode of pathogenic action with that of certain strains of the virus of febrile herpes which we also possess. As in our hands the Levaditi strain has shown itself not to be more, but actually to be less encephalitogenic than certain true herpes strains, we are reporting our experiments because of their bearing on current notions of the etiology of epidemic encephalitis.

Sources of the Herpes Virus.

We have studied a considerable number of specimens of the herpes virus as obtained from different persons, and have determined that they show wide variations of activity when inoculated into rabbits.[3] Two strains have been distinguished from the rest as possessing high power to infect rabbits. One of these two strains has been chiefly used in carrying out the many experiments to be reported on, while the other has been employed chiefly as a check upon the former. The H. F. strain, the one principally studied, was taken from a fresh herpetic vesicle on the lip of a subject very prone to attacks of febrile herpes. The clear, straw-colored fluid was drawn into a Luer syringe containing sterile salt solution and injected intracranially into anesthetized rabbits. The fluid proved to be bacteriologically sterile. The injected rabbits developed typical symptoms of virus

[3] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli (in press).

encephalitis to which they succumbed. With this virus (brain) a long series of inoculations of the rabbit has been carried on.

The study of the H. F. virus has extended over a period of nearly 3 years, during which high activity has been maintained not only in direct passages but also after long glycerolation. The H. F. virus has been used in parallel tests with the J. B. virus as well as with Levaditi virus *souche* C, and a specimen of Doerr or Basel virus.[4] Moreover, a few comparative tests have been made with Goodpasture Strain M virus.[5] We are greatly indebted to Dr. Levaditi and Dr. Goodpasture, and Professor Doerr for kindly providing us with specimens of their viruses.

As our purpose in this paper is primarily to show that the H. F. virus is markedly neurotropic and, at the same time, readily implantable upon the cornea and skin of the rabbit, we shall content ourselves with recording illustrative protocols only. In the interest of brevity it may be stated here that the inoculation of the H. F. virus provokes in the rabbit those typical symptoms and associated histological lesions and cell inclusions now accepted as characteristic of the herpes and encephalitic kinds of virus.

Intracranial Inoculation.

Experiment 1. Suspension Inoculation.—From Mar. 23 to Apr. 14, 1922, the H. F. virus was passed through a successive series of four rabbits, by intracranial inoculation.

Rabbit 1. The original diluted vesicular contents inoculated. Violent symptoms appearing on the 6th day, the animal was etherized. The brain, which was congested, was sterile in ordinary cultures.

Rabbit 2. Inoculated from Rabbit 1. Violent and characteristic symptoms and death on the 4th day. Brain cultures sterile.

Rabbit 3. Inoculated from Rabbit 2. Symptoms and death on the 2nd day. Cultures of brain sterile.

Rabbits 4 and 5. Inoculated from Rabbit 3. Symptoms leading to death on the 6th day within 12 hours of each other.

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[4] As the fact may prove of interest in the future, we desire to record that in our hands the Doerr strain of virus has been less intensively and regularly active than even the Levaditi strain. Moreover, it has shown itself of low comparative glycerol resistance. In these two respects it resembles certain weak herpes strains studied by us.[3]

[5] Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923–24, xliv, 121.

Rabbits 6 and 7. Inoculated from Rabbit 5. Symptoms leading to death on the 4th and 5th days respectively.

In this series of rabbits the outstanding symptoms included torpor, hyperpyrexia, gnashing and salivation, tremor, convulsions, paralysis, urine retention, and head and shoulder turning. The sections of the brain examined microscopically showed the usual infiltrative lesions of the meninges and tissues, as described in the preceding paper.[6]

From the glycerolated brain tissues of these rabbits other rabbits were inoculated intracranially 6 or 7 months later, evoking the characteristic symptoms, among which may be especially mentioned an irregular, backward movement or walk, rhythmical erection and extension of the body on the hind legs, leading to a kind of somersaulting. Following the fall backward the animals became wildly excited, running or walking rapidly in circles, the direction always being toward the inoculated side.

The H. F. strain of virus, having been shown effective by direct intracranial injection of fresh and glycerolated tissue, was next tested by means of (*a*) paper and (*b*) Berkefeld filtrates.

It is common practice to speak of the active brain tissue as "virus." Of course, this tissue is merely the carrier of the real virus which has been implanted upon it. When the brain is emulsified and suspended in salt solution, a part of the virus goes over into the fluid. This part is less concentrated than the original tissue, and hence tends to infect more slowly, or after a greater incubation period. In a like manner the Berkefeld filtrate carries a less concentrated virus than does the paper filtrate. Because of this difference in concentration a Berkefeld filtrate may prove to be ineffective, while the paper filtrate from which it was made is still active. This difference is more likely to appear when glycerolated instead of fresh virus (brain) is employed for the inoculation. We have already pointed out that glycerolation may reduce the potency of the virus (number of viable germs) below the frankly infective, but not beyond the protective capability. Berkefeld filtration, on the other hand, diminishes the potency further below the latter threshold.[6]

Experiment 2. Paper Filtration.—Glycerolated virus (brain) is made into a 5 per cent suspension in salt solution by trituration with sterile sand, shaking for 30 minutes, centrifugalization at slow speed for 3 minutes, and filtration through triple folded chemical paper.

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[6] Flexner, S., and Amoss, H. L., *J. Exp. Med.*, 1925, xli, 215.

Three rabbits were injected intracranially respectively with 0.01, 0.05, and 0.10 cc. of filtrate. All developed symptoms and all succumbed on the 3rd day.

The test was repeated in two rabbits with a specimen of virus which had been glycerolated for 6 months. Both animals showed characteristic symptoms and died on the 8th day.

Berkefeld Filtration.—Three separate tests of Berkefeld filtrates, prepared from glycerolated virus, failed to produce infection. A later test with such a filtrate of fresh virus (brain) gave an unequivocal positive result. From the brain of the animal thus infected a second Berkefeld filtrate was prepared and inoculated intracranially, likewise with a positive result.

Distribution of Virus in Central Nervous System.

The obvious lesions of experimental virus encephalitis, as far as they are known, arise in the forebrain, midbrain, and medulla; the spinal cord is visibly affected far less often. And yet the virus itself is detectable, by inoculation tests, throughout the central nervous system. It is of some interest, in view of the difficulty experienced in separating old or non-virus lesions of the brain from the true virus lesions[6] to find this lack of topographical relationship between site of lesions and presence of virus. The conditions in this repect simulate those of poliomyelitis in man and monkey, except that the predilection site of the lesions is reversed. While in poliomyelitis the spinal cord and medulla are visibly chiefly attacked, yet the virus is present in all parts of the central nervous organs.

Experiment 3.—May 26, 1923. A rabbit received intracranially 0.35 cc. of a 10 per cent suspension of glycerolated virus. Typical symptoms appeared and the animal was etherized on the 5th day. The brain was sterile for ordinary bacteria. June 2. Six rabbits were injected intracranially with a suspension from the brain of the preceding animal: two with tissue taken from the extreme forebrain, two from the medulla and cerebellum, and two from the dorsal and lumbar cord. All six rabbits showed the typical symptoms and all succumbed.

Corneal, Skin, and Nasal Inoculation.

Samples of herpes virus of diverse origin provoke, as a rule, fatal encephalitis when injected intracranially into rabbits. The case is different with inoculations into the surface structures of the body —the cornea, skin, and nasal mucosa. Hence, through the use of superficial (ectodermal) inoculations, distinctions of neural affinity may be detected.

The virus does not attack the unbroken skin of the rabbit, but it may be implanted on the cornea, or even on the nasal mucosa which has not been intentionally abraded. Probably, however, the procedures employed to bring the virus into the eye and nose suffice to produce microscopic injury to the delicate tissues.

The common experimental method is to smear the shaven and, often, injured (incised) skin surface with brain pulp carrying the virus, to instill a suspension of the pulp into the cocainized eye, in which superficial incisions have been made with the cataract knife, and to smear the pulp gently on the upper nasal mucosa, or to carry it there on a cotton plug. Ordinary inflammatory effects are thus produced, which, in respect to the skin and cornea, are open to inspection. Hence, beside suppuration, vesiculation is detectable. Probably vesiculation arises also in the nasal mucous membrane.

Besides these local effects, deeper and more severe ones often declare themselves. They consist precisely of those symptoms denoting an inflammation of the brain. It is with respect to this power, especially, to provoke virus encephalitis upon superficial inoculation that the various strains of herpes virus differ among themselves. Certain strains tend but little, other strains regularly, to this eventuality. With the former strains, also, the encephalitis induced inclines toward a milder clinical course from which recovery may ensue. With the latter recovery almost never occurs. The H. F. virus belongs in the latter class. Moreover, it is so potent that it may be said that superficial inoculation invariably leads to encephalitis followed by death. Hence it is appropriate to designate this strain as "encephalitogenic." The encephalitis-provoking propensity of the H. F. strain exceeds that of any of the so called epidemic encephalitis strains of virus (Levaditi, Doerr) at our disposal, is equalled perhaps by the J. B. strain,[6] isolated by us, and is approached, although probably not equalled, by the Goodpasture M herpes strain.[5]

Corneal Inoculation.—

Two rabbits were inoculated as follows: The left eye was cocainized for 20 minutes and then lightly scarified with a cataract knife dipped in a 10 per cent fresh brain (virus) suspension. The right eye was likewise cocainized and scarified, but not inoculated. Jan. 1, 1923. The left eye was closed by purulent inflammation. The right eye was uninflamed. Jan. 5 and 6. One animal

showing severe symptoms of virus encephalitis and turning to the left and falling over; the other also showing symptoms and drawing head backward and to the left side. Both rabbits succumbed.

Two additional rabbits were inoculated as above, in the left cornea. Suppuration and vesiculation followed and symptoms of virus encephalitis, including circling to the left and leading to death, resulted.

Glycerolation of the brain (virus) does not, as a rule, mitigate the effects, or change the result of the corneal inoculation. An occasional glycerolated specimen will, however, prove to have deteriorated and to have become wholly inactive. It has also been observed that the H. F. virus attacks the cornea vigorously merely after cocainization and without any scarification whatever.

May 19, 1923. The left eye of a rabbit was cocainized for 20 minutes, after which 1 drop of a 10 per cent suspension of fresh virus (brain) was lightly placed in the eye. Keratoconjunctivitis, followed by severe general symptoms and death on the 12th day, ensued. Among the symptoms was the characteristic circling to the side of the inoculated eye. With the brain of this rabbit a second animal was successfully inoculated into the cocainized eye, and with the brain of the second a third and a fourth animal were equally successfully inoculated. All the rabbits so treated developed first marked suppurative keratoconjunctivitis, then severe general symptoms of virus encephalitis, following which death ensued.

The strictly intact or normal eye does not respond to the instillation of the virus with keratoconjunctivitis. A fleeting redness (mild inflammation) alone results. Obviously, therefore, the cocaine solution induces changes in the cornea and conjunctiva of sufficient magnitude to enable the virus to become attached to and to multiply in those structures.

To recapitulate: Fifteen rabbits received instillation of H. F. virus into the cocainized eye, either with or without coincident scarification of the cornea. Eleven were inoculated with the fresh and four with the glycerolated virus. All the rabbits developed severe keratoconjunctivitis, succeeded by symptoms of virus encephalitis, and succumbed.

As will be apparent from the foregoing, the H. F. strain of virus is readily implanted upon the prepared eye of the rabbit and, without exception, when so implanted, invades the brain, bringing about a fatal issue. Since, recently, Levaditi has contended for a particular

encephalitogenic property of certain strains of herpes virus, derived especially from cases of epidemic encephalitis in man, it is fortunate that, through his kindness, we possess a sample of his variety (*souche*) C virus to which he ascribes in high measure this peculiar effect. In our hands the Levaditi virus has acted irregularly when inoculated into the eye after cocainization and scarification of the cornea. Of four rabbits, in which a keratoconjunctivitis of greater or less degree of severity was induced with this strain, in one only did symptoms of virus encephalitis and death supervene.

Skin Inoculation.—The abraded skin of the rabbit readily responds to herpes virus inoculation. Whether so slight a lesion as that produced in the eye by cocaine suffices to render implantation on the skin possible is not known. What is readily demonstrable is that the lightest shaving of the skin makes it vulnerable. After mere shaving, or shaving plus superficial incising, the smearing of brain pulp (virus) over the skin surface suffices to induce inflammation, vesiculation, and later desquamation. Not every rabbit so treated reacts typically, since a mild dermatitis sometimes results which, from subsequent immunity tests, appears not to be herpetic in nature.

The H. F. virus proved highly effective when inoculated into the skin. A certain difference was again noted between the effects of fresh and of glycerolated virus. The fresh virus may be regarded as producing almost without exception vesicular dermatitis, and almost invariably setting up, on the heels of this process, a fatal virus encephalitis. The glycerolated virus, on the other hand, behaves somewhat irregularly: either no dermatitis at all is produced, or a milder, specific inflammation arises, which does not lead to virus encephalitis. That this milder skin affection is really herpetic in nature is shown later by the immunity which has been excited by the process. The explanation of this somewhat anomalous effect is, we believe, to be found not in a mitigated H. F. virus as such, but merely in a numerical reduction in the implanted germs. In support of this belief is the oft observed fact that, when the same specimen of glycerolated virus is injected intracranially, it proves as virulent as ever, and the brain of the succumbing rabbits as active as ever upon skin implantation. Illustrative protocols follow.

Two rabbits inoculated with fresh virus, one upon the upper lip and gum, the other on the shaved and scarified skin of the abdomen. In both, pustulation and vesiculation occurred, and in both, on the 7th day, general symptoms, followed by death, ensued.

Four rabbits were inoculated with fresh virus over the shaved and scarified skin of the abdomen. All developed local and general symptoms and all died.

Four other rabbits were very lightly shaved with a particularly sharp razor over the abdomen and fresh virus was applied. Three developed mild, local, but severe, general symptoms and succumbed. The fourth animal showed neither local nor general effects and, on subsequent test, proved not to be immune to corneal inoculation of the virus.

Of thirteen rabbits inoculated on the skin surfaces with fresh brain (virus), eleven developed dermatitis, followed by virus encephalitis and death. Two rabbits did not respond at all and were susceptible to a second inoculation of the H. F. virus, to which they succumbed. In general it may be stated that the greater the local, traumatic injury, the severer the local inflammation. But the power of a strain of herpes virus to invade the body and set up encephalitis is independent of its ectotropic property. Not all strains of herpes virus which excite skin (or corneal) inflammation induce a coincident encephalitis. Hence, by the skin test also, the H. F. virus merits being considered encephalitogenic.

Intranasal Inoculation.—Cotton swabs carrying active virus were inserted into the nares and left in contact with the mucosa of four rabbits for 12 hours. All four animals developed symptoms of virus encephalitis and succumbed on the 6th to the 9th day following the inoculation.

In three other instances milder, less encephalitogenic strains of virus were introduced in the same way into the nares.[3] Symptoms of much less severity followed, from which the animals recovered. These rabbits, on being subsequently tested, proved to be immune.

Intravenous and Intratesticular Inoculation.

In view of the power displayed by the H. F. virus to penetrate from superficial structures into the body, and thus to provoke encephalitis, it was to be supposed that, when introduced directly into the blood, a similar effect would follow; and the same was predicated

for the injection when made into the testicle, in which organ it is known that the virus survives and probably multiplies. The accepted notion is that the herpes virus, like the virus of poliomyelitis, is capable of ascending to the central nervous organs by way of the peripheral nerves.[7] Probably also, when active and in adequate quantity, the virus passes the barrier of the choroid plexus, thus again acting in the manner of the virus of poliomyelitis. The matter of host susceptibility plays, however, an essential part in this regard. Not only does the rabbit respond to the intracranial injection of the herpes virus, but certain other rodents—guinea pig, rat, mouse,—respond also. But no other rodent than the rabbit becomes infected when the herpes virus is introduced into the blood or the testicle.

As is to be expected, the symptoms and lesions arising from the intravenous and intratesticular injections of the virus in rabbits are identical with those contingent upon other modes of inoculation. In no instance did the fresh virus fail to induce characteristic symptoms and to bring about the death of the rabbits when injected either into the blood or the testicle.

Infection of Other Rodents.

Guinea pigs, rats, and mice were given intracranial injections of fresh virus (rabbit brain) with the effect of inducing severe symptoms of virus encephalitis terminating in death. When, however, identical samples of the virus were injected into the heart or the testicle, encephalitis did not follow and the animals showed no symptoms of note.

In general, it may be stated that the symptoms of virus encephalitis in the smaller rodents mentioned agree with those observed for the rabbit. Certain distinctions have, however, been noted: in the guinea pig, paralysis rather than excitation, and in the rat and mouse muscular excitement rather than depression and paralysis are the rule. It was not unusual for the guinea pigs to salivate, become tremulous, ataxic, convulsive, and then paralytic, while the rats and mice often executed complex, rapid, rhythmical movements, not

[7] Goodpasture, E. W., and Teague, O., *J. Med. Research*, 1923–24, xliv, 139.

observed at all in the guinea pig, and in a less degree of intensity in the rabbit. The circling movements of the rats and mice were sometimes bewilderingly rapid; they were, because of the intrinsic interest, recorded in moving picture films.

SUMMARY.

In this paper we have sought to show that unequivocal strains of herpes virus exist in man, which, in the rabbit, exhibit a degree of encephalitogenic power not exceeded, and perhaps rarely equalled, by any strain of the so called encephalitis virus.

The fact that such highly encephalitogenic strains of the herpes virus exist in nature has, at the moment, theoretical and practical importance. Until recently, the view has been accepted by certain workers in the field that two biologically distinct viruses of this class occur—one inducing epidemic encephalitis and the other febrile herpes in man.

This view, is, indeed, being supplanted at the present time by the notion, advocated by Levaditi, Nicolau, and Poincloux, of a group of closely related virus organisms for which the name "herpetico-encephalitic" is proposed. Within this group they distinguish strains of virus displaying special affinities for the central nervous organs and others exhibiting equal affinities for skin and membrane (cornea) structures. The first mentioned strains are responsible, under suitable circumstances, for epidemics of encephalitis in man; the others give rise to ordinary attacks of febrile herpes.

The H. F. virus described in this paper does not conform to the classification indicated. While being a true febrile herpes strain, it possesses, nevertheless, a high degree of power to attack the central nervous system as well as marked capacity to implant itself on the skin and the cornea of the rabbit. Not only does virus encephalitis follow invariably upon the intracranial injection of the H. F. virus, but as regularly upon corneal, skin, nasal, blood, and testicular modes of inoculation. The symptoms of virus encephalitis thus provoked and the character of the brain lesions induced are precisely those, in all their detail and variety, including the presence of intracellular inclusion bodies, which have been described for the so

called virus of encephalitis. Moreover, the H. F. virus is durably glycerol-resistant, is filterable through Berkefeld candles, and behaves immunologically as do the usual strains of herpes and of encephalitis virus.

On the basis of the experimental data presented, we conclude that any distinction made regarding, on the one hand, encephalitogenic power as a special property of a virus secured from cases of epidemic encephalitis, and, on the other hand, of ectotropic action as an equally special quality of a virus yielded by febrile herpes, is in its nature artificial and not in harmony with ascertained fact. What can, indeed, be distinguished are stronger and weaker strains of a virus, probably always herpetic in origin, as determined by the inoculation of rabbits. While a strong herpes virus is both dermatotropic and neurotropic, a weak virus tends, in its multiplication, to remain confined to the site of inoculation, to act chiefly on the tissues on which it is immediately implanted, and not to extend to distant parts. And this is equally true whether the strain of virus came originally from cases of epidemic encephalitis, or merely from cases of febrile herpes in man.

Hence direct comparison cannot be made between the stronger encephalitogenic and weaker non-encephalitogenic strains, according to any specific etiological property. The viruses we are discussing do, indeed, compose one group but it is the group of febrile herpes with which epidemic encephalitis is associated accidentally, if at all. It happens, indeed, that the Levaditi strain (*souche*) C and the Doerr Basel strain, both supposedly originating in cases of encephalitis in man, are less encephalitogenic for the rabbit than the true herpes strains, H. F. and Goodpasture M.

A COMPARISON OF THE EFFECTS OF X-RAY AND DRY HEAT ON ANTIBODY FORMATION.

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The location within the body of antibody formation has remained undetermined in spite of extensive investigation. However, much of the collected data points to the lymphoid tissue as importantly concerned therein.

The significant point in this relation is the fact that antibodies during immunization are found earlier and in greater concentration in the spleen than in the blood serum,[1] and the further observation that removal of the spleen in the early stages of immunization causes a retardation in antibody production.[2-4] The results of splenectomy have failed to convince, as the severity of the operation alone might easily account for the falling off in antibody production. The fact that animals immunized after recovery from splenectomy develop antibodies much as do normal animals has little of value for it is well known that other lymphoid structures quickly compensate for the loss of the spleen.

Hektoen,[4,5] in an attempt to throw light on the subject, has utilized the fact that x-ray produces a destructive effect on the lymphoid structures, and has shown that antibody formation is restrained to a marked degree in x-rayed animals. He concluded that antibodies are formed in the spleen, lymphatic tissue, and bone marrow since these are the structures most affected by x-ray.

[1] Pfeiffer, R., and Marx, *Z. Hyg. u. Infectionskrankh.*, 1898, xxvii, 272. Wassermann, A., *Berl. klin. Woch.*, 1898, xxxv, 209. Castellani, A., *Z. Hyg. u. Infectionskrankh.*, 1901, xxxvii, 381. Cantacuzène, J., *Ann. Inst. Pasteur*, 1902, xvi, 522. Tsurumi, M., and Kohda, K., *Z. Immunitätsforsch., Orig.*, 1913, xix, 519.

[2] Deutsch, L., *Ann. Inst. Pasteur*, 1899, xiii, 689.

[3] Hektoen, L., *J. Infect. Dis.*, 1909, vi, 78.

[4] Hektoen, L., *J. Infect. Dis.*, 1920, xxvii, 23.

[5] Hektoen, L., *J. Infect. Dis.*, 1915, xvii, 415; 1918, xxii, 28.

Our own earlier studies have developed the facts (1) that x-ray, in properly graded doses, depletes the lymphoid tissues without damage to the bone marrow,[6] and (2) that dry heat stimulates the activity of those organs.[7] It therefore seemed to us that in these two contrary effects on the lymphoid structure of the body, we possess means of determining quite directly the part they play in antibody formation.

The plan we pursued was the following. Extensive preliminary experiments were carried out in order to determine the number and intensity of x-ray doses required to reduce the amount of lymphoid tissue in rabbits without damage to the bone marrow. The method eventually evolved was to give from three to five daily exposures to x-ray, the dosage being governed by the following factors: spark-gap 3 inches, milliamperes 10, target distance 6 inches. The duration of the exposure was 4 minutes, 2 to the upper half of the animal and 2 to the lower. This series of three or more daily exposures was given prior to or immediately following the first injection designed to elicit antibodies and was repeated weekly throughout the experiments. For the stimulation of the lymphoid structures the rabbits were given a 15 minute exposure to dry heat at a temperature ranging from 50–52°, one exposure before the immunizing injections were started and others thereafter at weekly intervals. Particular care was taken, in working out the dosage of these two physical agents, that the treatments caused no loss of weight or other evidence of interference with the general health of the animals.

Precipitin Formation.[8]

Experiments 1 to 3.—Since practically the same methods were used in these three tests they will be reported together. The experimental groups were made up of (1) rabbits depleted by x-ray, (2) normal rabbits, and (3) rabbits stimulated by

[6] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459. Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397. Murphy, Jas. B., and Taylor, H. D., *J. Exp. Med.*, 1918, xxviii, 1. Taylor, H. D., Witherbee, W. D., and Murphy, Jas. B., *J. Exp. Med.*, 1919, xxix, 53.

[7] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1. Nakahara, W., *J. Exp. Med.*, 1919, xxix, 17.

[8] We wish to express our appreciation to Dr. O. T. Avery for material help in the planning and carrying out of the experiments reported in this paper.

dry heat. All of these animals were injected with 10 cc. of horse serum intravenously 1 or 2 days after the first series of x-ray treatments and 3 or 4 days after the heat exposure. A second injection of 5 cc. of horse serum was given 1 week later and a third of 2 cc. after still another week. The heat and x-ray treatments were continued at weekly intervals throughout the experiment. All of the rabbits were bled from the heart 14 days after the final injection of antigen, and the serum was separated. For each of the sera to be tested a set of tubes was prepared containing 0.5 cc. of the various dilutions of the antigen. To each of these was added 0.5 cc. of the rabbit serum and the tubes placed in a water bath at 37°C. for 2 hours, after which a preliminary reading was made. The final observation was recorded after 12 hours in the ice box. Suitable controls were carried for each test, consisting of horse serum with salt solution, the individual rabbit sera, both with salt solution and with several dilutions of guinea pig serum. The results are given in Table I.

While the difference in potency of the precipitin in the sera from the three groups is striking, the difference in the amount of precipitate formed is even more so. In the majority of instances the amount of precipitate thrown down by the sera from the heated animals in antigen dilutions of 1:4,000 was equal in amount to that formed in dilutions of 1:40 from the control rabbits.

Bacterial Agglutinins and Protective Antibodies.

For the following experiments we have used Pneumococcus Type I for the antigen and the technique for immunization in use at the Hospital of The Rockefeller Institute.[9]

Experiments 4 and 5.—In Experiment 4, three groups of rabbits were prepared in the same manner as in the above described experiments; *i.e.*, one group with the lymphoid tissue reduced by repeated exposure to x-ray, another group stimulated by dry heat, and a third made up of untreated animals. A series of five daily x-ray exposures was given in this experiment instead of three as in the previous ones, and the immunizing injections were started 2 days prior to the first x-ray and heat applications. Experiment 5 was similar except that no x-rayed animals were included. As the technical procedures were the same the two experiments will be described together.

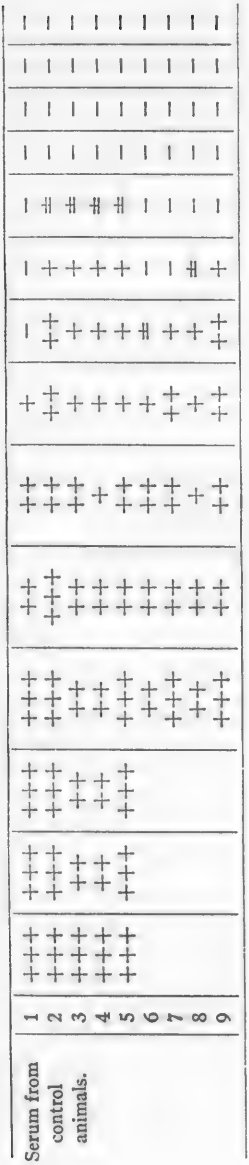
All of the rabbits received six daily intravenous injections of 1 cc. of a Type I pneumococcus vaccine, consisting of heat-killed organisms suspended in a sufficient amount of physiological salt solution so that 1 cc. was equal in bacterial count to 1 cc. of the original culture. 7 days later the rabbits were bled from the

[9] Cole, R., and Moore, H. F., *J. Exp. Med.*, 1917, xxvi, 537.

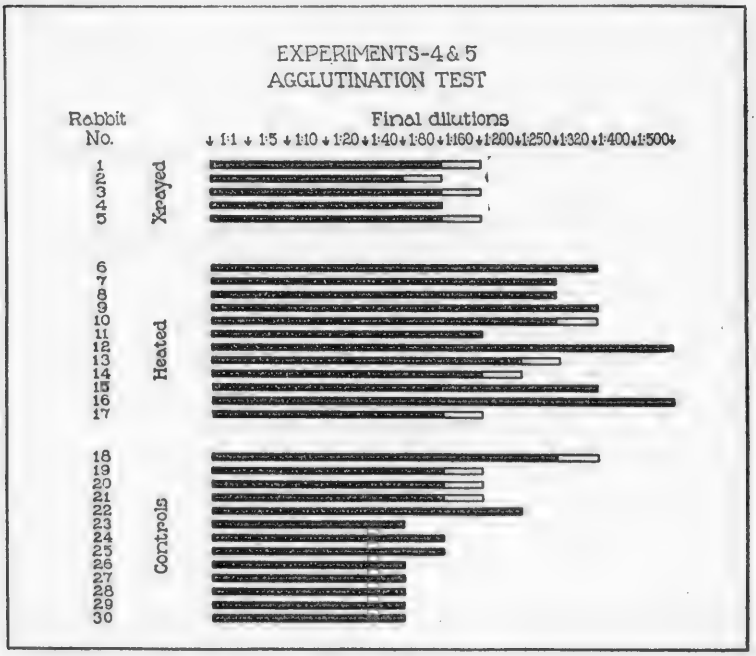
TABLE I.

Precipitin Value of Sera from X-Rayed Rabbits, Heated Rabbits, and Controls.

		Antigen dilution.												
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,000	1:2,000	1:4,000	1:8,000	1:16,000	1:20,000
		Final dilution.												
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,000	1:4,000	1:8,000	1:16,000	1:32,000	1:40,000	Controls.
Serum from x-rayed animals.	1	+++	++	++	+	−	−	−	−	−	−	−	−	−	−
	2	++	++	+	−	−	−	−	−	−	−	−	−	−	−
	3	++	++	++	++	++	+	−	−	−	−	−	−	−	−
	4	++	++	++	++	++	+	−	−	−	−	−	−	−	−
	5	++	++	++	+	+	+	+	−	−	−	−	−	−	−
	6				±	±	−	−	−	−	−	−	−	−	−
	7				−	−	−	−	−	−	−	−	−	−	−
	8				+	+	±	±	−	−	−	−	−	−	−
	9				++	++	+	+	+	−	−	−	−	−	−
Serum from heated animals.	1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	−	−	−
	2	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−	−	−
	3	++++	++++	++++	+++	+++	++	++	++	++	++	++	+	−	−
	4	++++	++++	++++	+++	+++	+++	++	++	++	++	+	+	−	−
	5	++++	++++	+++	+++	+++	+++	+++	+++	++	++	++	+	−	−
	6	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	−	−	−
	7				+++++	+++++	+++++	++++	+++	+++	++	+	±	−	−
	8				++++	++++	++++	+++	+++	++	+	+	+	±	−
	9				++++	++++	++++	+++	+++	++	+	+	+	+	−
	10				+++	+++	+++	+++	++	+	+	+	±	−	−


Serum from control animals.
1
2
3
4
5
6
7
8
9

marginal vein of the ear and the agglutinating power of the sera was tested. After the elapse of this 7 day rest period a second series of six daily injections of vaccine was given, and then, after yet another rest period a third series of six injections was made. Throughout the experiment the heat treatments were repeated at weekly intervals and the course of five x-ray exposures was repeated every 2nd week. 9 days after the last vaccine dose all of the rabbits were bled from the heart and the sera collected.



TEXT-FIG. 1.

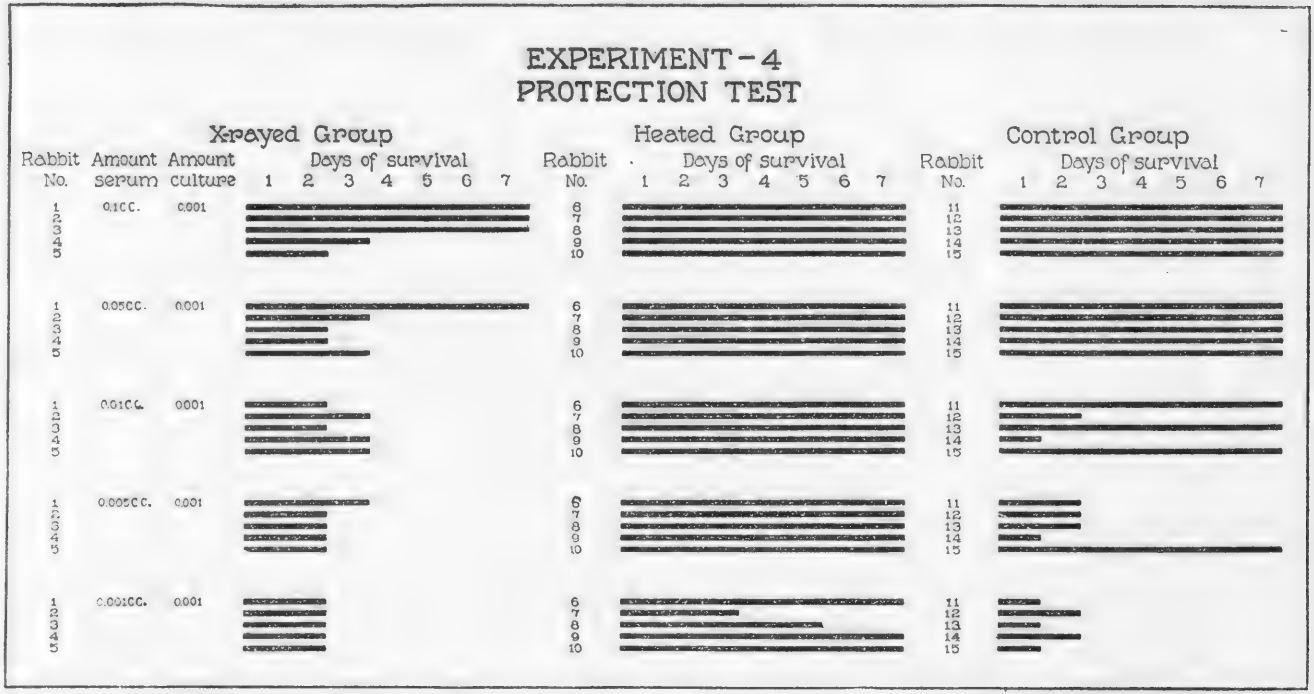
Agglutinins.—To test-tubes containing 0.5 cc. of the various sera diluted so as to give a range of from 1:5 to 1:250, was added 0.5 cc. of a suspension of Type I pneumococcus. After a thorough shaking the tubes were placed in a water bath at 37.5°C. for 2 hours and then in the ice box overnight. The final readings made at this time are recorded in Text-fig. 1.

Protective Antibodies.—The sera from all of the rabbits were also tested for their protective power against infection. A Type I pneumococcus of the Neufeld strain was subjected to two animal passages, thereby increasing its virulence to a

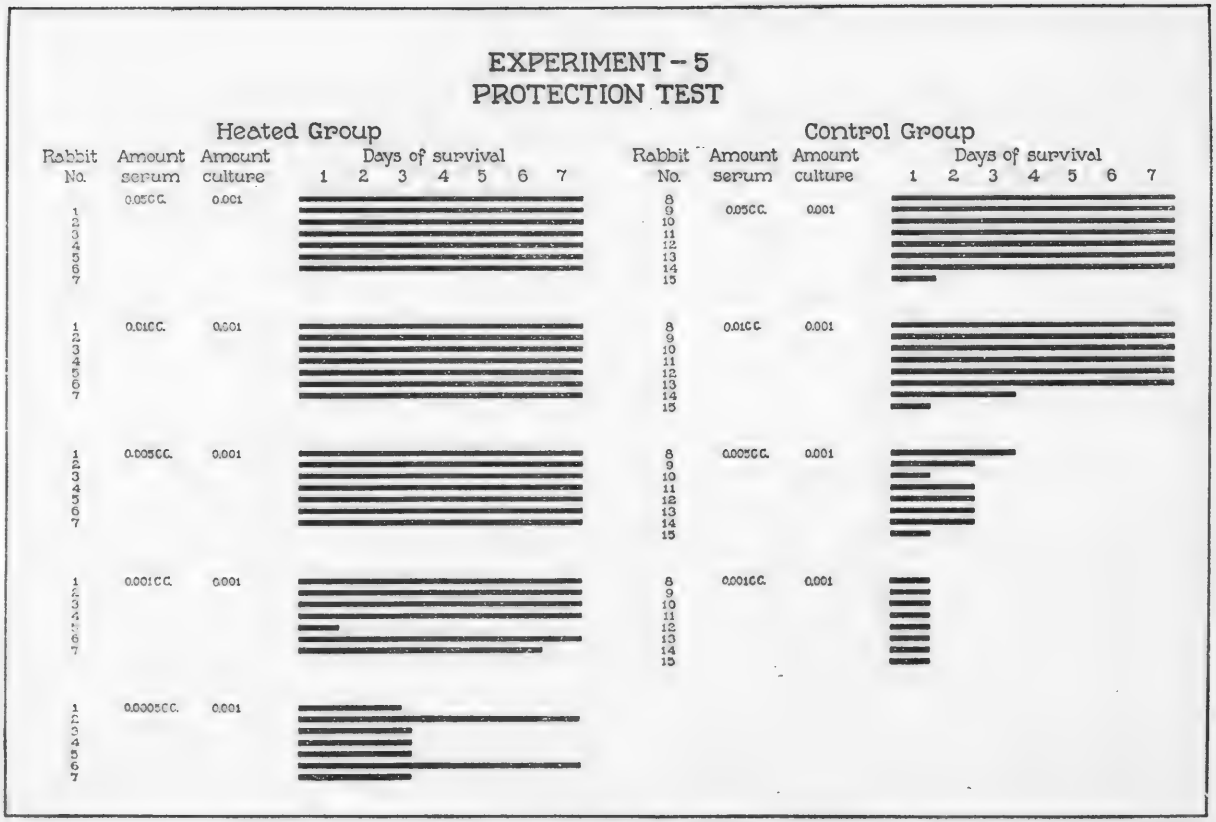
point where 0.000001 of a 12 hour broth culture killed mice within 48 hours and 0.001 uniformly caused death within 18 hours. For each serum to be tested for its protecting power, six normal white mice weighing from 18 to 20 gm. were given an intraperitoneal injection of 0.001 of a 12 hour bouillon culture of the organism mixed with graded amounts of the immune sera ranging from 0.1 to 0.001 cc. In some instances, when the serum protected in these amounts, additional tests were carried out with smaller amounts. Mice which survived for 7 days were considered effectively protected from infection. The results of this experiment are given in Text-figs. 2 and 3.

The agglutinating power of the sera from the three groups of rabbits, even in specimens taken after the first series of vaccine injections, showed distinct differences. The titer of the sera from the heated rabbits was almost uniformly higher and that from the x-rayed rabbits lower than in the case of the controls. In the final test, the sera from two heated animals agglutinated in dilutions as high as 1:500 with the average for the group of about 1:320. On the other hand, sera from the control rabbits failed to agglutinate in dilutions above 1:80 in nine out of thirteen specimens and in only one instance did agglutination take place in dilutions higher than 1:200. The sera from the x-rayed group was somewhat less potent than that from the controls but not strikingly so. From these results it would appear that the heat treatment not only gives rise to an increase in the amount of circulating antibodies but also increases the rate of production.

The protection test demonstrated quite as clearly the difference in the strength of the sera of the three groups. That from the x-rayed animals was not uniformly protective even in 0.1 cc. amounts, whereas the sera from the controls gave little protection when less than 0.01 cc. was used. On the other hand, 0.005 cc. of sera from heated rabbits uniformly protected the mice against infection, and as little as 0.001 cc. of eight out of the twelve specimens was found to be sufficient. Two of these sera gave complete protection even when so small an amount as 0.0005 cc. was injected with the infecting organism. In other words, the heat treatment effected a tenfold increase or an even greater one in the rabbits' ability to develop protective antibodies, whereas the x-ray treatment has inhibited the production to about an equal amount.


EXPERIMENT-4
PROTECTION TEST
X-rayed Group
Heated Group
Control Group
Rabbit No.
Amount serum
Amount culture
Days of survival
1 2 3 4 5 6 7
0.1CC.
0.05CC.
0.01CC.
0.005CC.
0.001CC.
0.001

TEXT-FIG. 2.


EXPERIMENT – 5
PROTECTION TEST
Heated Group
Rabbit No.
Amount serum
Amount culture
Days of survival
1 2 3 4 5 6 7
0.05 C.C.
0.001
0.01 C.C.
0.001
0.005 C.C.
0.001
0.001 C.C.
0.001
0.0005 C.C.
0.001
Control Group
Rabbit No.
Amount serum
Amount culture
Days of survival
1 2 3 4 5 6 7
0.05 C.C.
0.001
0.01 C.C.
0.001
0.005 C.C.
0.001
0.001 C.C.
0.001

TEXT-FIG. 3.

DISCUSSION.

The experiments here reported confirm and extend the observations of Hektoen and others that x-ray in large doses given before and during the immunizing process retards definitely the production of such antibodies as precipitins, bacterial agglutinins, and protective antibodies. The present work shows that x-ray will effect this retardation when given in such a manner as not to damage the bone marrow while seriously injuring the lymphoid tissue. The evidence pointing to the lymphoid tissue as the manufacturer of antibodies is strengthened by the further observation that an animal's ability to form such bodies may be greatly increased by the stimulation of the lymphoid tissues by dry heat.

There are not sufficient data at hand to justify final deductions from Gay and Clark's[10] observation that animals vitally stained with trypan blue develop antibodies with extreme slowness. This fact these authors interpret as evidence that the reticulo-endothelial system, which is principally affected by the dye, is the site of antibody formation. It is true that the endothelial cells of the lymphoid organs following destructive doses of x-ray are engorged with the remains of the lymphoid cells, but granted that such engorged cells are incapacitated for antibody formation, the total number of them in the lymphoid system represents such a minute fraction of the total number in the animal body that their incapacity would scarcely explain the results of our experiments. Furthermore, there is no evidence that dry heat affects endothelial cells in the slightest.

Gay and Clark make the point in favor of the reticulo-endothelial system as the source of antibodies that its wide distribution throughout the body would account for the phenomenon of local immunity. The same argument may be presented in favor of the lymphoid tissue, for small deposits of this tissue are scattered throughout the body and it is well known that the small local accumulations may be quickly augmented either by multiplication of the existing cells or by migration from the blood vessels.

While the lymphoid organs would seem to be concerned in the responses which we have detected in the course of the present work the

[10] Gay, F. P., and Clark, A. R., *J. Am. Med. Assn.*, 1924, lxxxiii, 1296.

possibility cannot be excluded that other organs and physiological processes are influenced by the x-ray and heat and affect the results. At the moment we have no way of settling this matter. With this limitation we can conclude that along with the manifest changes in the lymphoid organs, of depletion on the one and stimulation on the other hand, there are correlated decreases and increases in the production respectively of precipitating, agglutinating, and protecting antibodies.

SUMMARY.

Rabbits x-rayed in doses sufficient to reduce the amount of their lymphoid tissue without damage to the bone marrow showed a definite deficiency in the production of precipitins, bacterial agglutinins, and protective antibodies. On the other hand, rabbits subjected to exposures of dry heat sufficient to increase the activity of the lymphoid organs, on immunization develop antibodies in larger quantity than do untreated animals immunized by the same process.

THE INFLUENCE OF THE "DIAPLYTE" ANTIGEN OF DREYER ON TUBERCULOSIS OF THE GUINEA PIG.

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(Received for publication, October 14, 1924.)

Recently Dreyer[1] has developed a method whereby tubercle bacilli can be rendered, more or less readily, non-acid-fast. The non-acid-fast bacterial residue has, according to this author, proved capable not only of stimulating the production of specific antibodies in normal and in tuberculous animals, but has yielded promising therapeutic results in experimental tuberculosis. Since the original experiment of Dreyer included few animals, while the infection with which he dealt was of a decidedly mild type possibly permitting spontaneous recovery in some instances, it has seemed desirable to repeat the work.

Preparation of Antigen.

Through the courtesy of Dr. William H. Park and Dr. Charles Krumwiede we obtained a mass culture of tubercle bacillus Ravenel on glycerol broth and proceeded to prepare the antigen according to the method of Dreyer. A mass of bacilli is ground in a mortar, in the presence of small amounts of formalin, until a thick, homogeneous paste is obtained. This is diluted by the further addition of formalin to even emulsion containing 150 to 200 cc. of formalin for each 5 gm. of bacteria (weighed wet). This emulsion is placed in a flask fitted with a reflux condenser and heated in boiling water for 4 hours. The bacteria are then filtered off, washed freely with acetone, and subjected to a prolonged (20 to 24 hours) extraction with acetone at 65–70°C. in a Soxhlet apparatus. If, at this stage, the organisms still show acid-fastness when stained in the usual way, they are again subjected for 3 to 4 hours to treatment with formalin and subsequently to extraction with acetone. This alternate treatment with formalin and acetone is repeated until the organisms have been rendered non-acid-fast. In our experience the process has had to be repeated four times. The bacteria

[1] Dreyer, G., *Brit. J. Exp. Path.*, 1923, iv, 146.

thus divested of their acid-fast envelop ("diaplyte") still retain their bacillary outline and take the counterstain. 0.1 gm. of such diaplyte bacterial mass is then thoroughly ground in a sterile agate mortar and little by little suspended in physiological (0.9 per cent) salt solution so that at the end of the grinding, the original 0.1 gm. of bacteria is represented by 10 cc. of a fine suspension. This is subjected to brief centrifugation at high speed (about 3,000 revolutions per minute) until the larger particles have all disappeared from the supernatant fluid, as determined by the examination under No. 6 objective. The fluid is now further diluted with sterile physiological salt solution containing 0.15 per cent formalin so that, with proper correction for the coarser particles of bacillary substance removed by centrifugation, 1 cc. of the final dilution would contain *0.2 mg.* of bacterial substance. This fine suspension was stored in the ice box and used for the treatment of animals.

Series 1.

Infection of Animals.—Guinea pigs were used exclusively for our experiments. No effort was made to select the animals according to sex or age, but as a rule the animals weighed about 350 to 450 gm. at the beginning of the experiment. Having established the virulence of the culture by preliminary tests, we inoculated 99 animals by a subcutaneous route with a homogeneous suspension of tubercle bacillus (Ravenel) in physiological salt solution. This suspension had been prepared by careful trituration for an hour of a weighed amount of bacteria with physiological salt solution, added drop by drop. When the total volume of the suspension had reached about 15 cc., it was briefly centrifuged at high speed and the supernatant fluid, comparatively free from clumps and containing an even suspension of bacteria, was diluted with salt solution so that, after due correction for the amount of bacteria removed by centrifugation, each cc. of suspension contained approximately 0.1 mg. of bacteria. The amount inoculated was selected with a view to initiating a comparatively slow process and yet one calculated not to allow the animals to remain alive so long that spontaneous healing might take place in the more resistant individuals and thus confuse the results. To lessen the likelihood of discharging sinuses following inoculation, we first inserted a sterile 3½ inch needle under the skin and then attached to it the syringe loaded with the bacterial suspension. In this manner each animal was given 1 cc. of suspension containing 0.1 mg. of bacteria. Following withdrawal of the needle, the site of puncture was thoroughly swabbed with iodine.

The animals, thus inoculated, were subsequently separated into four groups —A (21), B (23), C (21), and D (34) respectively (Chart 1). 3 days after inoculation, all animals in Group A received subcutaneously their first dose of antigen containing an equivalent of 0.1 mg. of the defatted bacterial residue. About 2 weeks later the administration of vaccine was begun with the animals of Group B and, after another interval of 2 weeks, with the animals of Group C.

Those of Group D received no antigen whatsoever and served as controls. A summary of the general plan of the experiment is given in Chart 1.

From the date of infection, the animals were frequently weighed and examined. General appearance and condition, local and general reactions to

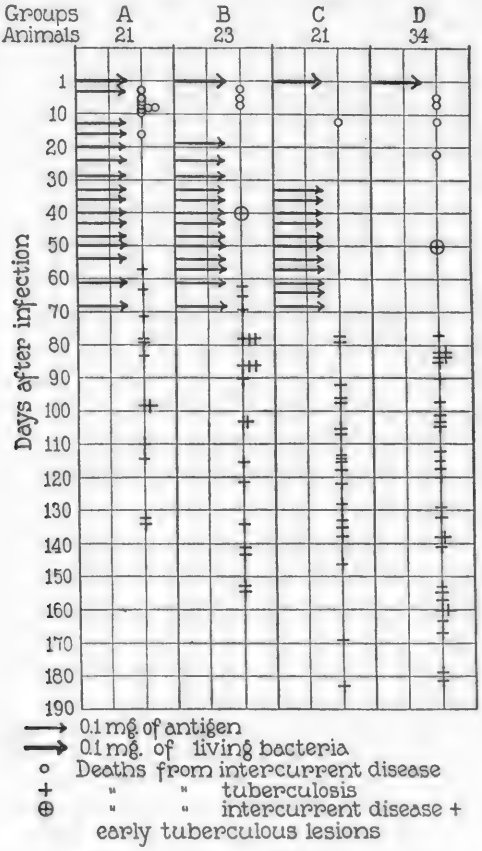

CHART 1. Summary of Experiment 1.

treatment, and the development of adenopathies were all carefully noted and individual records were maintained for each animal.

The treatment with diaplyte antigen, once started, was repeated once or twice weekly over a period of about 10 weeks as shown in Chart 1. We did not observe any local or general reactions immediately following the injections.

In all, during the course of the experiment, the animals of Group A received fifteen injections of the diaplyte antigen; those of Group B, thirteen such injections; and those of Group C, eleven.

Unfortunately, during the first 2 weeks, the animals were kept in rather crowded quarters and several guinea pigs from the various groups succumbed

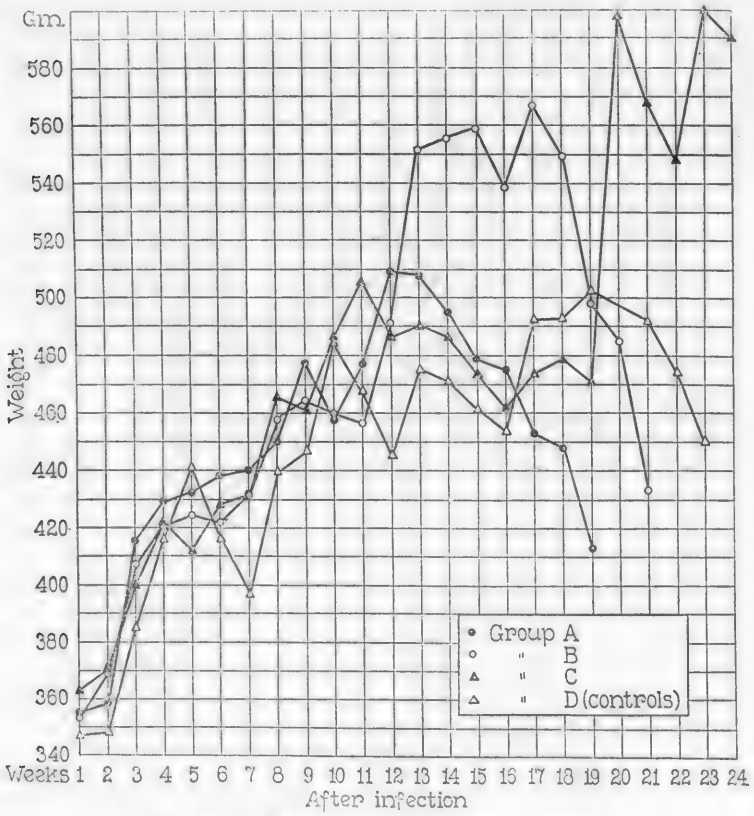

CHART 2. Weekly weight averages by groups.

to intercurrent infections. Animals of Group A suffered the most, with nine deaths out of the total number of twenty-one animals. Groups D, B, and C were affected in the order named with four, three, and one deaths respectively (circles on Chart 1). Upon a change in housing conditions, however, the animals seemed to thrive better. Nevertheless, two more of them—one in Group B

and the other in Group D—died during the course of the experiment from other causes than tuberculosis, although at postmortem early tuberculous lesions were already discernible in both (cross within circle, Chart 1).

Weight Curves.—Since in his paper Dreyer stressed the beneficial effect of treatment as interpreted by the weight curve of his animals, we have attempted,

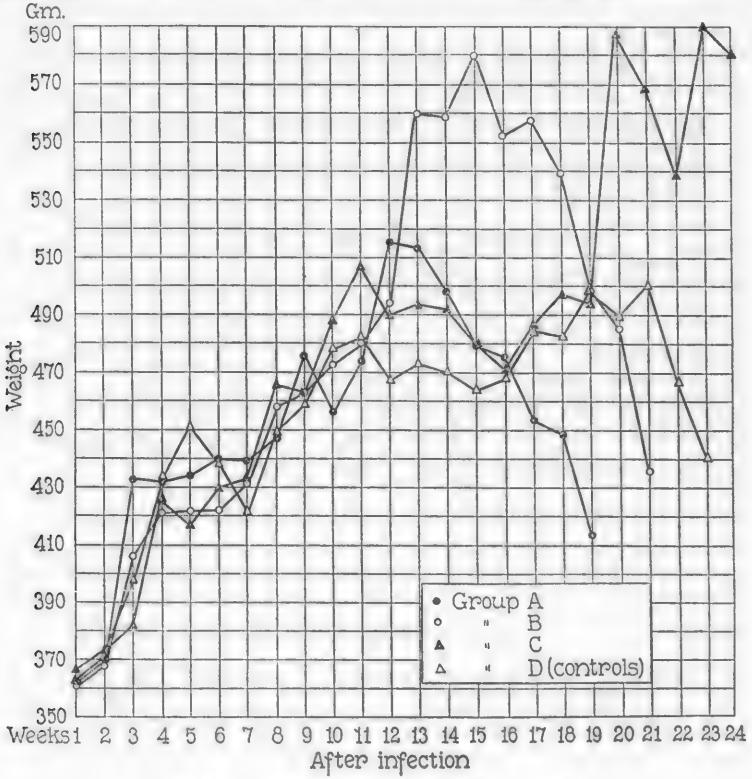

CHART 3. Weekly weight averages of males.

in Chart 2, to compare the weights of the animals of the different groups, by superimposing the average curves as made up from the individual weekly weights of the animals in each group over the entire period of the experiment.

In order to eliminate the effect upon average curves of change in weight during pregnancies, developing in the course of the experiment, we excluded pregnant animals from the calculation of averages as soon as we could diagnose the condition. The weights of these animals after the termination of gestation were

again included in the calculation. The method is inexact, but since we were purposely dealing with both sexes, housed together and under similar conditions the inaccuracy is difficult to overcome. In Charts 3 and 4, a further analysis of weight curves, from the point of view of the influence of sex, is attempted.

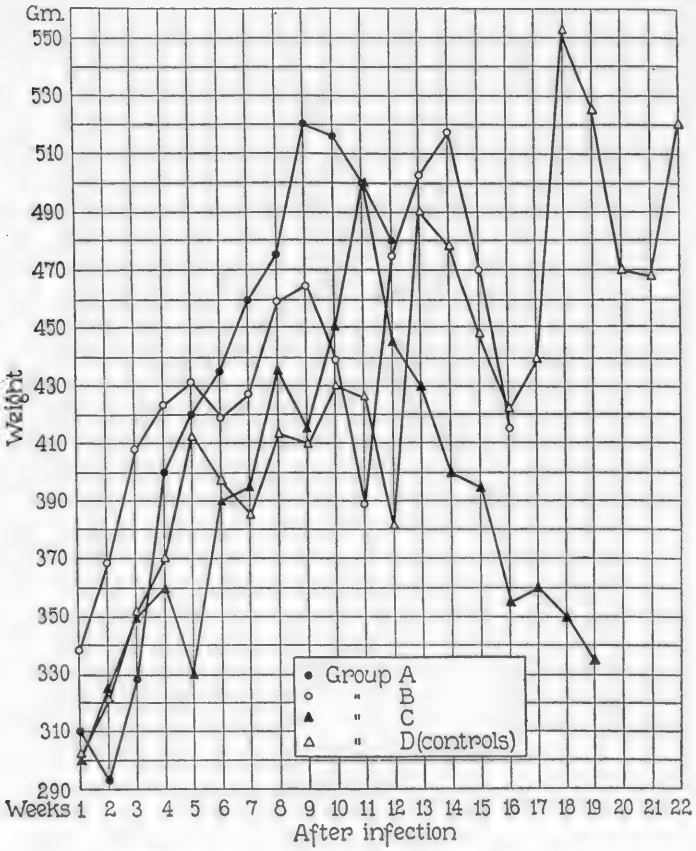

CHART 4. Weekly weight averages of females.

It will be seen that the curves for females show variations in the different groups for the reason just dealt with, while the group curves for males closely resemble the original curves, in which both sexes are considered together (Chart 2).

In general, the treatment appeared to make no difference in the average weight of the animals during the first 16 weeks, during which time the individual variations in the resistance of animals to infection were submerged because of the large number of animals entering into the calculation. But as the experiment continued and the number of surviving animals in each group became smaller, the curves showed the result of selection of resistant individuals, as in the case of Group B and especially in Group C, which latter from the 19th week of the experiment was represented merely by a single very resistant male survivor. The number of animals alive at any time can be calculated from Chart 1.

Postmortem Findings.—8 or 9 weeks after the inoculation, deaths from tuberculosis began to occur in all groups (crosses on Chart 1). In each case, a postmortem examination was performed and the gross findings recorded. In addition, smears from the affected organs were stained and examined for tubercle bacilli.

In as far as could be determined from gross examination of the autopsy material, the character and distribution of the lesions have been in general similar in the animals of all the groups. No attempt was made to study the microscopic character of the lesions.

The lymph nodes throughout were as a rule involved to a greater or less degree. This was especially true in the case of the tracheobronchial nodes,—a characteristic feature of generalized tuberculosis in guinea pigs, no matter how infection of these animals is induced.[2–4]

The lungs and pleuræ were found to be affected in greater or lesser degree, as result apparently of individual variation. There were no characteristic differences among the groups. The heart was usually free from lesions, although the pericardium was frequently found to be adherent in animals showing tuberculous pleurisy.

Marked individual variations were to be noted in the extent of involvement of the abdominal organs. Usually there were to be found more or less extensive lesions in the liver and spleen. And, in a certain number of animals, there was present extensive tuberculous peritonitis with dense adhesions and frequently with marked ascites.

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[2] Krause, A. K., *The Harvey Lectures*, 1921–22, xvii, 128.

[3] Rogers, J. B., *Am. Rev. Tuberc.*, 1919–20, iii, 750.

[4] Baldwin, E. R., and Gardner, L. U., *Am. Rev. Tuberc.*, 1921–22, v, 429.

Other organs, such as the intestines, kidneys, genitalia, etc., were involved only occasionally.

In the course of examination of the brains, undertaken in another connection by Dr. Cowdry, occasional tubercle bacilli were demonstrable in the tissue of animals from all four groups.

The gross autopsy findings yielded no evidence of differences between the condition of animals treated with antigen and the untreated controls.

Rate of Progress of Infection.—Although all the animals were inoculated at one time and in the same manner, Chart 1 suggests that deaths from tuberculosis appeared earliest among the animals of Group A. Mortality in the other groups seemed to follow the

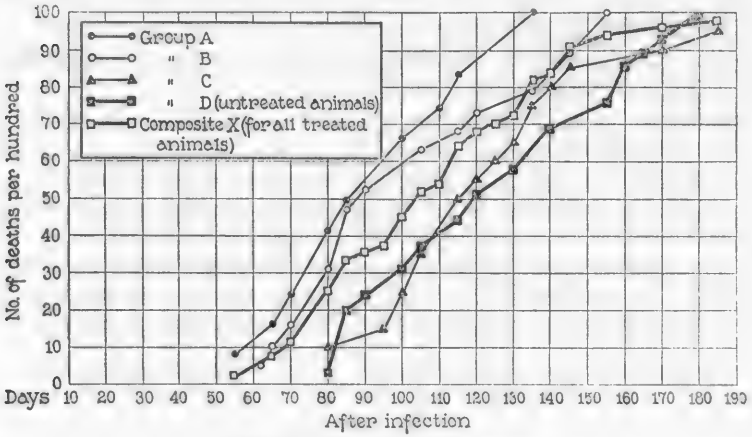

CHART 5. Death rate by groups.

order in which treatment had been instituted, namely B, C, and lastly D,—the control group receiving no therapeutic injections. This difference in the occurrence of first deaths might conceivably have been accidental. On the other hand, if it held true throughout the experiment, it would indicate that the treatment influenced the course of the disease in the direction opposite to that found by Dreyer.

Inquiring into this question, we compared the death rates in different groups.

To eliminate the effect of losses from early intercurrent infections,

the calculation of the death rates from tuberculosis was made on the basis of totals obtained by subtracting the number of deaths from intercurrent infections in each group from the original number of animals used; thus the total number for Group A was taken to be 12 instead of 21, for Group B 19 instead of 23, for Group C 20 instead of 21, and for Group D 29 instead of 34. The points on the curves in Chart 5 express the percentage relationship of dead animals to this corrected total. In addition to the four curves representing the death rates of animals in Groups A, B, C, and D, we plotted a fifth curve (X) which represents the composite death rate of all treated animals, irrespective of the time of beginning of treatment.

The data from which the curves were plotted are given in Table I.

As can be seen from Curve X in Chart 5 the effect of treatment was to accelerate the fatal issue rather than to delay it, as might have been expected from Dreyer's results. Group C, composed of animals in which the therapeutic injections were begun last, had a death rate nearest to Curve D of the controls. The other two curves (Groups A and B) suggest that the earlier the treatment was begun the more rapid was the progress of the disease to a fatal termination.

As already stated, we lost, shortly after beginning the experiment, over 15 per cent of our animals from intercurrent infection. Not knowing how successful we might be in checking further losses, we decided to repeat this experiment at once, in order to save time. But in planning the new experiment, we introduced slight modifications so as to obtain additional information, with regard especially to the possible prophylactic value of Dreyer's antigen.

Series 2.

In this experiment the attempt was made to immunize twenty guinea pigs prior to infection (see Group F in Chart 6). After five injections of standard suspension of diaplyte antigen in the course of 17 days, we inoculated them, together with 56 untreated animals, by a subcutaneous injection of 0.05 mg. of an even suspension of living tubercle bacilli in physiological salt solution. The strain of tubercle bacillus, the method of preparation of bacterial suspension, and the method of injection of the suspension were the same as had been employed in the preceding experiment, with the exception that the suspension used for infection of the animals in this experiment contained 0.05 mg. of bacteria per cc. instead of 0.1 mg., as before.

The 56 unimmunized inoculated animals were now separated into three groups. The twenty animals of Group G were given, beginning 9 days after infection, nine injections of 0.1 mg. of diaplyte antigen, with about 4 days interval between successive injections. The guinea pigs of the second group,

TABLE I.

Death Rate of the Animals in Experiment 1.

Group A. Original No. of animals 21. Corrected No. of animals 12.			Group B. Original No. of animals 23. Corrected No. of animals 19.			Group C. Original No. of animals 21. Corrected No. of animals 20.			Group D (controls). Original No. of animals 34. Corrected No. of animals 29.			Composite curve X for all treated animals. Original No. of animals 65. Corrected No. of animals 51.		
Days after infection.	Total No. dead.	Per cent of total (approximate).	Days after infection.	Total No. dead.	Per cent of total (approximate.)	Days after infection.	Total No. dead.	Per cent of total (approximate).	Days after infection.	Total No. dead.	Per cent of total (approximate).	Days after infection.	Total No. dead.	Per cent of total (approximate).
55	1	8.33	62	1	5.25	80	2	10.0	80	1	3.45	55	1	1.96
65	2	16.66	65	2	10.50	95	3	15.0	85	6	20.70	62	2	3.92
70	3	24.99	70	3	15.75	100	5	25.0	90	7	24.15	65	4	7.84
80	5	41.65	80	6	31.50	105	7	35.0	100	9	31.05	70	6	11.76
85	6	49.98	85	9	47.25	115	10	50.0	105	11	37.95	80	13	25.48
100	8	66.64	90	10	52.50	120	11	55.0	115	13	44.85	85	17	33.32
110	9	74.97	105	12	63.00	125	12	60.0	120	15	51.75	90	18	35.28
115	10	83.33	115	13	68.25	130	13	65.0	130	17	58.65	95	19	37.24
135	12	100.00	120	14	73.50	135	15	75.0	140	20	69.00	100	23	45.08
			135	15	78.75	140	16	80.0	155	22	75.90	105	27	52.92
			145	17	89.25	145	17	85.0	160	25	86.25	110	28	54.88
			155	19	100.00	170	18	90.0	165	26	89.70	115	33	64.68
						185	19	95.0	170	27	93.15	120	35	68.60
						278	20	100.0	180	29	100.00	125	36	70.56
												130	37	72.52
												135	42	82.32
												140	43	84.28
												145	46	90.16
												155	48	94.08
												170	49	96.04
												185	50	98.00
												278	51	100.00

designated in Chart 6 as Group H and consisting of twenty animals, received six injections of antigen, beginning 19 days after infection; and Group I, comprising sixteen animals, received no treatment (control).

These animals weighed between 250 and 300 gm. at the beginning of the experiment. Sex was not considered in their selection.

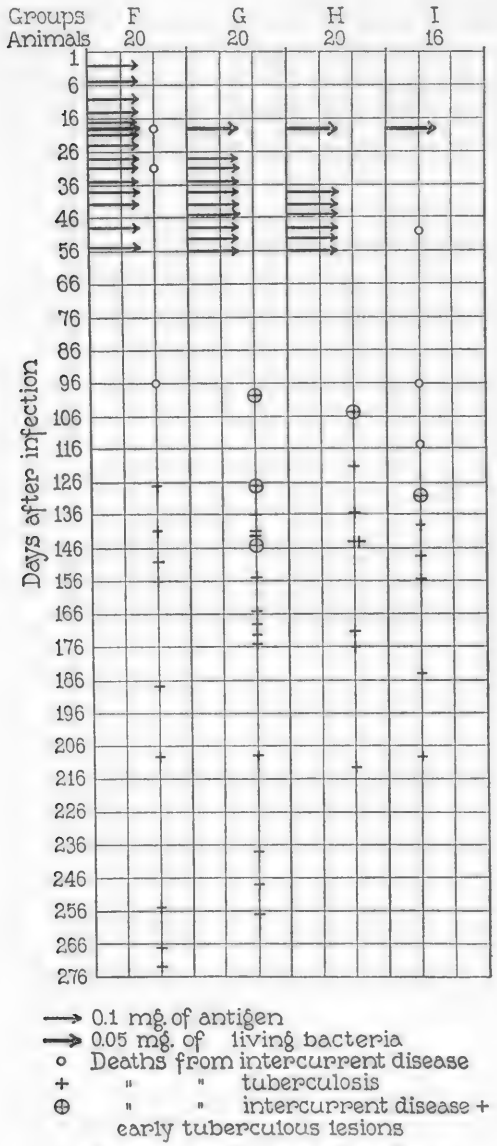

CHART 6. Summary of Experiment 2.

Weight Curves.—As in the previous experiment, we attempted to judge the relative progress of infection, in the animals of the several groups, by comparing the average weight curves, plotted in the manner already described. Charts 7 to 9 cover the first 22 weeks of the experiment and plainly indicate that, as in the preceding experiment, there was no marked difference in the weight curves of the groups.

TABLE II.

Death Rate of the Animals in Experiment 2.

Group F. Total No. of animals 20. Corrected No. of animals 17.			Group G. Total No. of animals 20. Corrected No. of animals 20.			Group H. Total No. of animals 20. Corrected No. of animals 20.			Group I (controls). Total No. of animals 16. Corrected No. of animals 13.			All treated animals (Y). Total No. of animals 60. Corrected No. of animals 57.		
Days after infection.	No. dead.	Per cent.	Days after infection.	No. dead.	Per cent.	Days after infection.	No. dead.	Per cent.	Days after infection.	No. dead.	Per cent.	Days after infection.	No. dead.	Per cent.
110	1	5.9	80	1	5.0	85	1	5.0	110	1	8.0	80	1	2.0
125	2	12.0	110	2	10.0	100	2	10.0	120	2	15.0	85	2	4.0
130	3	18.0	115	3	15.0	115	3	15.0	130	3	23.0	100	3	5.0
135	4	24.0	120	5	25.0	125	5	25.0	140	4	31.0	110	5	9.0
170	5	29.0	130	6	30.0	150	6	30.0	165	5	40.0	115	7	12.0
190	6	35.0	135	7	35.0	160	7	35.0	190	6	46.0	120	9	16.0
235	7	41.0	145	8	40.0	195	8	40.0				125	12	21.0
250	8	47.0	150	9	45.0							130	14	25.0
255	9	53.0	155	11	55.0							135	15	26.0
			190	12	60.0							140	16	28.0
			220	13	65.0							145	17	30.0
			230	14	70.0							150	18	32.0
			240	15	75.0							155	21	37.0
												160	22	39.0
												170	23	40.0
												190	25	44.0
												195	26	46.0
												220	27	48.0
												230	28	49.0
												235	29	51.0
												240	30	53.0
												250	31	54.0
255	9	53.0	255	15	75.0	255	8	40.0	255	6	46.0	255	32	56.0

Mortality.—At the present time, about 57+ per cent of the animals have died. Of this number, about 75 per cent died from tuberculosis, about 12 per cent showed generalized tuberculous lesions at autopsy, but probably died from intercurrent disease, while the

remainder succumbed early from intercurrent disease and showed no tuberculous lesions at autopsy. From the data recorded in Table II and plotted in Chart 10, it is apparent that the death rate among the controls was no more marked than in the treated animals. In fact, from present appearances, the control animals seem more resistant to infection than some of the treated ones.

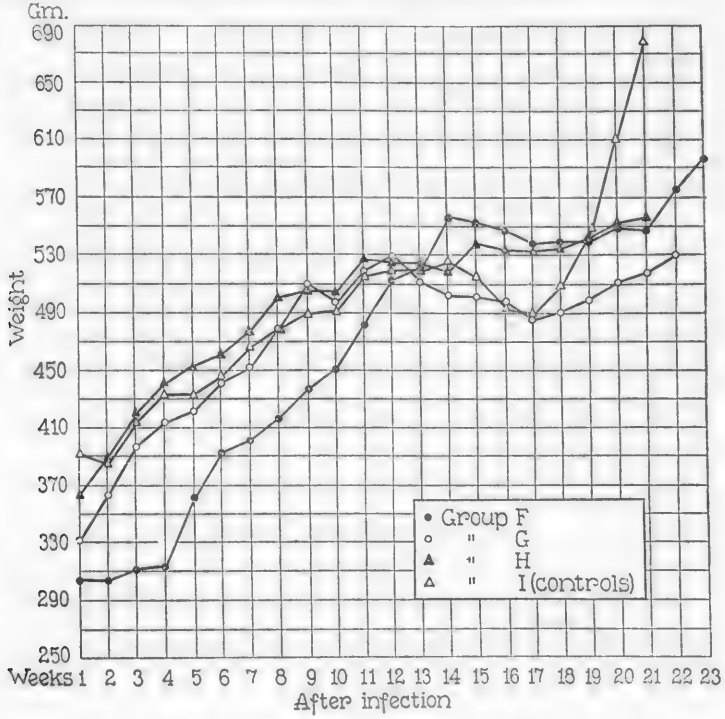

CHART 7. Weekly weight averages by groups. Experiment 2.

In as far as the individual groups are concerned, Group F shows 53 per cent mortality to date, Group G 75 per cent, Group H 40 per cent, and Group I (controls) 46 per cent.

Accessory Experiments.

Upon consideration of the foregoing results it seemed possible that the test of resistance to which we were subjecting our animals was perhaps too severe.

With this in mind, we planned two small experiments essentially similar to those already described, with the exception that the tubercle bacilli were introduced into the animals without trauma.

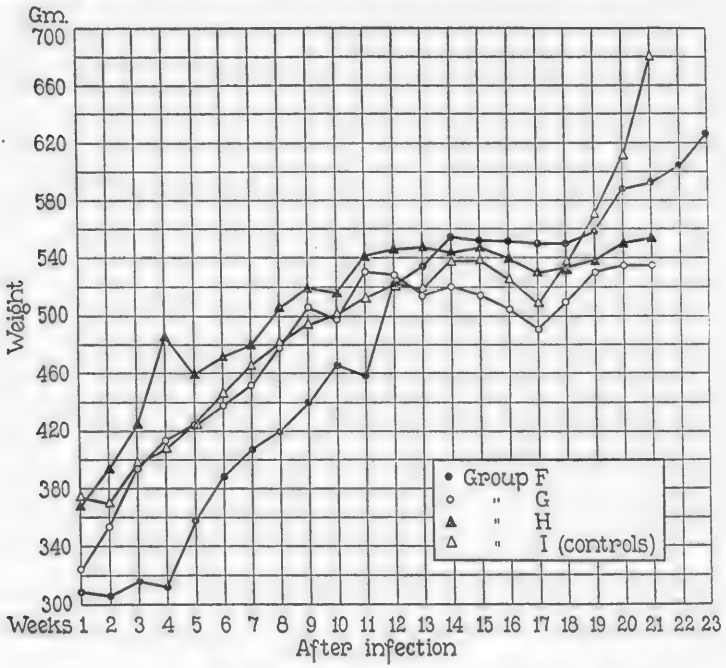

CHART 8. Weekly weight averages for males. Experiment 2.

Exposure to Cage Infection.

In this experiment ten guinea pigs, having received fifteen preliminary injections of Dreyer's antigen, with eight normal controls of similar weight, were housed in a large cage, with many frankly tuberculous guinea pigs. At the present time, about 9 months after the beginning of the experiment, two control animals out of the eight have succumbed to a generalized tuberculosis. Those still living show slight adenopathies. Of the ten animals immunized by Dreyer's

antigen five have died from advanced infection, and the remaining five have palpable adenopathies.

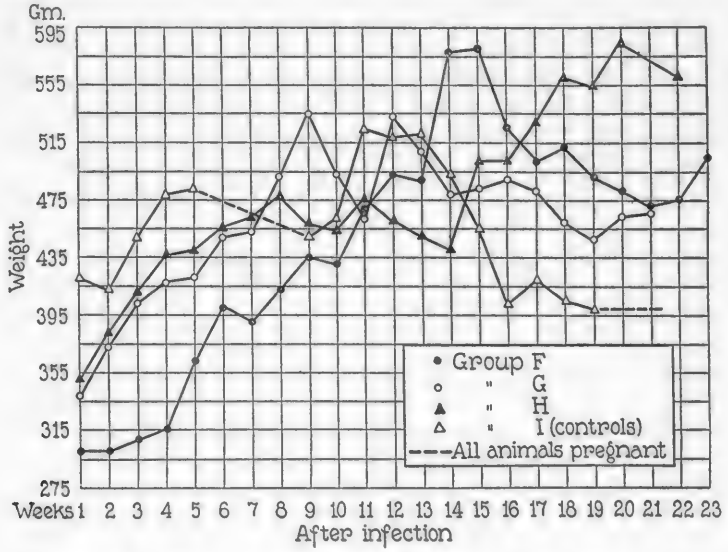

CHART 9. Weekly weight averages for females. Experiment 2.

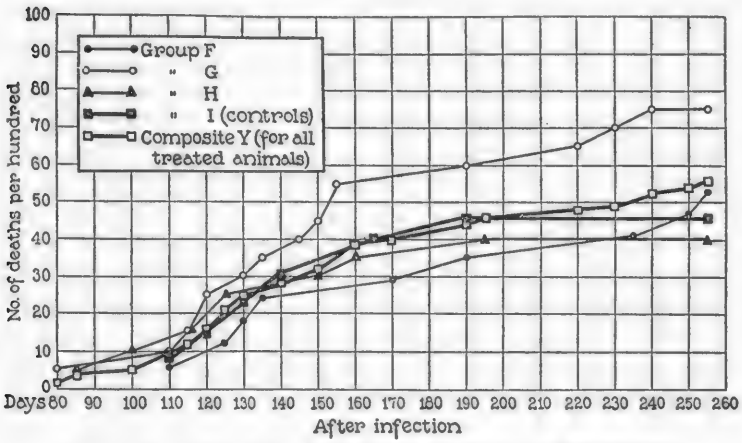

CHART 10. Daily average death rate by groups. Experiment 2.

Infection through the Conjunctiva.

This experiment comprised three groups of guinea pigs. One group, consisting of five, received a preliminary series of twelve subcutaneous injections of Dreyer's diaplyte antigen, at 4 to 6 day intervals. A second group, of four animals, received by the subcutaneous route seven preliminary injections of bacteria, defatted by the method of Fornet.[5] The third group (controls) consisted of four normal animals.

About 1 month subsequent to the last of the immunizing treatments, 0.02 mg. of tubercle bacilli in suspension was dropped into one conjunctival sac of each of the animals, with due precautions to avoid trauma. At the time of writing, about 4 months later, three of the four control animals are living, but all show definite adenopathies. One animal died about 11 weeks after the infection. Although death was probably not due primarily to tuberculosis, nevertheless at autopsy a picture of early generalized tuberculosis was presented. Three out of the four animals immunized with Fornet's antigen are living and present definite palpable adenopathies. One animal died a little over 4 months after infection. Autopsy showed the presence of a generalized tuberculosis. Finally, of five guinea pigs immunized with Dreyer's antigen, only one remains alive at the present time. The other four died between the 10th and 12th weeks after instillation of the bacterial suspension, from generalized tuberculosis.

The results of these experiments are tabulated in Table III.

TABLE III.

Results of Treatment in Animals Infected without Trauma.

	Group.*	Living.	Total dead.	Deaths from tuberculosis.	Deaths from intercurrent disease + tuberculosis.	Per cent deaths
Exposure to cage infection.	A. 10 animals treated with Dreyer's antigen.	5	5	3	2	50.0
	B. *8 normal control* animals.	6	2	2	0	33.0
Conjunctival instillation.	C. 5 animals treated with Dreyer's antigen.	1	4	3	1	80.0
	D. 4 animals treated with Fornet's antigen.	3	1	1	0	25.0
	E. *4 normal control* animals.	3	1	0	1	25.0

* Groups A and B were housed with frankly tuberculous guinea pigs. Groups C, D, and E received 0.02 mg. of bovine tubercle bacilli, per conjunctiva.

[5] Fornet, W., *Ann. Inst. Pasteur*, 1921, xxv, 797.

As in the earlier experiments, it here appears that the treatment with Dreyer's antigen contributed to the early development and rapid progress of infection as compared with the controls. Animals treated with the antigen of Fornet did not appear notably affected in resistance.

The results of the accessory experiments are too meager when considered alone to allow of conclusion, but we present them here because they yield evidence confirmatory to our previous, more extensive work.

DISCUSSION.

The data presented in the foregoing pages show clearly that in our hands Dreyer's vaccine has not conferred resistance to tuberculosis, when employed either as a prophylactic or as a therapeutic agent. Indeed a comparison of the average mortality curves for all the treated animals in each experiment with the curves of mortality of untreated controls would appear to indicate that the treatment might even diminish the resistance of guinea pigs to infection. This diminution in resistance is not very marked, but it is interesting to note that similar findings have recently been recorded by Kettle.[6]

In an effort to explain the failure to confirm Dreyer's findings several possibilities may be considered. In the first place, as stated earlier, Dreyer dealt with a small number of animals infected with an organism of very low virulence. The effect on the general results of high individual resistance becomes very marked under these conditions. For example, in our own series one guinea pig in a treated group (C) survived 9 months, 3 months longer than the other 98 animals. On the other hand, our failure might be attributable to some differing quality of the vaccine. However, Kettle, who used the antigen prepared by Dreyer himself, failed to observe any beneficial effects from it. Again, mistakes in duration and intensity of treatment might be held responsible for the failure on our part. It remains to be ascertained whether a different dosage may prove helpful in combating the disease. Riviere[7] has recently recommended a geometrical progression of dosage in the use of tuber-

[6] Kettle, E. H., *Lancet*, 1924, i, 68.
[7] Riviere, C., *Lancet*, 1923, ii, 324.

culins, including specifically Dreyer's antigen. The time when the therapy should be instituted is another uncertain point. We have noticed that animals which received their first treatment comparatively late after infection (Group C in Experiment 1 and Group H in Experiment 2) survived longer than the animals receiving early treatment. It is interesting to note in this connection that Dreyer first began his treatments 28 weeks after infection.

SUMMARY.

We have prepared "diaplyte" antigen according to Dreyer's procedure and have studied its therapeutic and prophylactic value in experimental tuberculosis of guinea pigs. In our hands it has failed to yield beneficial effects. The animals treated with the antigen tended in general to develop lesions more quickly and to die earlier than the controls.

EPIDEMIOLOGICAL STUDIES ON RESPIRATORY INFECTIONS OF THE RABBIT.

VI. Etiology of Otitis Media.

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(Received for publication, November 10, 1924.)

During the course of a number of autopsies on stock and experimental animals at The Rockefeller Institute, it was frequently noted that the middle ears contained pus. These observations led us to investigate otitis media as it occurs in rabbits with the purpose of determining its etiology and relation to other native infections and of gaining information which might possibly be useful in explaining similar conditions of the human ear.[1]

Occurrence.—In a series of consecutive autopsies on 68 experimental and 34 stock animals, 33 (32 per cent) were found to have middle ear infection. Of these, 31 had been used for experimental purposes and 2 came from supposedly normal stock (Table I).

Symptoms.—There are no outstanding characteristic symptoms of otitis media in the rabbit. Except for the relatively few instances in which pus is found in the external canal or the occurrence of torticollis and rotating movements, indicating an involvement of the vestibular apparatus, the diagnosis cannot be made before autopsy. Very frequently, however, animals with otitis have snuffles in either the acute or the chronic state and may lose weight and strength and finally die.

Gross Pathology.—Autopsies at various stages have shown that the progress of the infection is much like that exhibited by the human ear disease. In the first stage the tympanic membrane and the lining of the tympanic cavity are red. The cavity contains a serosanguineous fluid. Soon it becomes filled with thick, white, creamy

[1] Smith, D. T., *Am. J. Dis. Child.*, 1924, xxviii, 1.

pus. This may remain for weeks or months until it is gradually absorbed or until the animal is killed or dies of some intercurrent infection. At times, but less frequently than in the case of human otitis, the ear drum ruptures and pus is discharged into the external canal. This is generally followed by absorption of the remaining material and a healing of the ear drum with scar formation. When absorption has taken place one finds the membrane lining the cavity somewhat thickened and of a dirty grayish color.

Bacteriology.—Material from the middle ear, free from contamination, was obtained by the following technique.

The brain was first carefully removed with sterile instruments. Then, the inside of the skull was seared with a hot soldering iron and the middle ear opened

TABLE I.

		No. of rabbits.	Sinusitis.	Per cent.	Otitis.	Per cent.
Animals previously used for the study of	experimental syphilis	27	16	59	15	55
	" meningitis	16	7	44	7	44
	" tumor	14	3	21	5	35
	" thyroidectomy	10	3	30	3	30
	" dysentery	1	0	0	1	0
Total		68	29	42.6	31	45.5
Normal		34	3	8.4	2	5.0
Grand total		102	32	31.0	33	32.0

with a pair of sterile bone forceps. The tympanic cavity of the rabbit is quite large and it was relatively easy to get a loopful of pus from the center of the cavity without touching the walls or the broken surface of the petrous bone.

Smears and cultures were made from the pus found in the ears of twenty-five rabbits. The films were stained by Gram's method and generally showed large masses of pus cells, mainly of the mononuclear type, in various stages of degeneration. Bacteria were relatively few in number and were both extra- and intracellular.

Cultures were made by streaking a loopful of pus over the surface of a freshly prepared rabbit blood agar plate. When both ears were involved, each was cultured separately. Table II shows that *Bacterium lepisepticum* was isolated from twenty of the twenty-five animals. It was found in pure culture in thirteen and mixed with a diphtheroid organism or *Staphylococcus albus* in seven other cases.

Relation of Otitis Media to Infection of the Nasal Passages.—It was noted early that rabbits with snuffles very frequently showed otitis media also. Among the thirty-two animals with nasal infection, mentioned in Table I, twenty-five (78 per cent) of them showed evidence of middle ear involvement, and although an occasional rabbit with otitis showed no macroscopic lesion in the nasal passages, when a considerable number were examined the percentage incidence of both diseases was found to be practically the same.

TABLE II.

Bacteriology of the Otitis Media.

Bacterium lepisepticum	13
" " and *Staphylococcus albus*	4
" " " diphtheroid	3
Diphtheroid	2
" and colon-like organism	1
Gram-negative mucoid organism	2
Total	25

TABLE III.

Results of Cultures on the Nares and Middle Ear Cavities.

No. of rabbits.	*Bacterium lepisepticum* cultured.	Miscellaneous bacteria cultured.
9	Nares and ears.	0
3	Ears.	0
3	Nares and ears.	Diphtheroids from ears.
2	0	" " "
4	Nares and ears.	Staphylococcus " "
1	Ears.	*B. bronchisepticus* " "

These findings suggested the advisability of determining the bacteriological relationship between the infections. This was accomplished by culturing separately the nares and middle ear cavities of twenty-two rabbits which had both otitis and nasal passage involvement. Table III shows the results of these cultures. *Bacterium lepisepticum* was recovered from the nares and ears of fourteen animals, in pure culture from Nos. 1 to 9 inclusive, and in combination with diphtheroids or staphylococci from Nos. 13, 15, 18, and 19.

In seven cases (Nos. 10, 11, 12, 14, 20, 21, and 22) it was obtained from the ears but not from the nasal passages.[2] Two rabbits (Nos. 16 and 17) showed no *Bacterium lepisepticum* in either nares or ears.

Experimental Otitis Media with Cultures of Bacterium lepisepticum.—It was evident from the studies just described that *Bacterium lepisepticum* is the organism most frequently found in both otitis and nasal infections. Consequently, if an etiological relationship exists between these two conditions, cultures of this organism when introduced into the nasal passages of normal non-immune rabbits might be expected to induce both nasal and middle ear infections. An experiment was devised to test the supposition.

A group of young rabbits was available which had been reared under special conditions that precluded contact with *Bacterium lepisepticum* at any time previously. Into the nasal passages of eleven of these animals 1/4 cc. of a 16 hour culture of the organism was introduced. Clinical observations and subsequent autopsies and bacteriological cultures were made. Table IV summarizes the results. Three of the animals died of pneumonia; of the eight survivors, six developed snuffles and otitis and became carriers of the organism and one developed otitis media alone. Only one animal escaped infection.

Another group of nine animals of the same age and from the same source was treated in a similar way except that a preliminary attempt had been made to vaccinate them with killed and living cultures. The results are shown in Table V. Five came down with snuffles, these and two others became carriers, and all seven developed otitis media. From one case of otitis *Staphylococcus albus* alone was recovered, from the other six cases *Bacterium lepisepticum*. Two of the nine escaped infection with the organism used in the experiments. Although none of this series died of pneumonia, yet the percentage of middle ear infection was about as great as among the rabbits which had not been vaccinated.

Intranasal Inoculations with Bacillus bronchisepticus.—Because *Bacillus bronchisepticus* is found frequently in the nasal passages of stock rabbits and because it has been regarded by some as the cause

[2] This discrepancy may be due to an error in technique. Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 837, 843.

TABLE IV.

Outcome of Experimental Inoculation with Bacterium lepisepticum.

First Experiment.

Rabbit No.	Treatment.	Snuffles.	*Bacterium lepisepticum* carrier.	Pus in ears.		Culture of ears.
				Left.	Right.	
23	1/4 cc. *Bacterium lepisepticum* intranasally.	+	+	+	+	*Bacterium lepisepticum* both ears.
24	" "	+	+	+	0	" " left ear.
25	" "	+	+	+	+	" " both ears.
26	" "	+	+	0	+	" " right ear.
27	" "	+	+	+	+	" " both ears.
28	" "	+	+	+	+	" " " "
29	" "	0	0	+	+	" " " "
30	" "	0	0	0	0	Ears sterile.
31	" "	Died of pneumonia.				
32	" "	" " "				
33	" "	" " "				
Total.....	11	6	6	7		

TABLE V.

Outcome of Experimental Inoculation with Bacterium lepisepticum.
Second Experiment.

Rabbit No.	Treatment.	Snuffles.	*Bacterium lepisepticum* carrier.	Pus in ears.		Culture of ears.
				Left.	Right.	
34	1/4 cc. *Bacterium lepisepticum* intranasally.	+	+	+	+	*Bacterium lepisepticum* both ears.
35	" "	+	+	+	0	" " left ear.
36	" "	+	+	+	+	" " both ears.
37	" "	+	+	+	0	" " left ear.
38	" "	+	+	0	0	Ears sterile.
39	" "	0	+	+	+	*Bacterium lepisepticum* both ears.
40	" "	0	+	+	+	" " " "
41	" "	0	0	Serum.		*Staphylococcus albus* both ears.
42	" "	0	0	0	0	Ears sterile.
Total.....	9	5	7	7		*Bacterium lepisepticum* 6. *Staphylococcus albus* 1. Sterile 2.

TABLE VI.

Outcome of Experimental Infection with B. bronchisepticus.

Rabbit No.	Treatment.	Snuffles.	*B. bronchisepticus* carrier.	Pus in ears.	Cultures of ears.
43	1 cc. *B. bronchisepticus* intranasally.	0	+	0	Ears sterile.
44	" "	0	+	0	" "
45	" "	0	+	0	" "
46	" "	0	+	0	" "
47	" "	0	+	0	" "
48	" "	0	+	0	" "
49	" "	0	+	0	" "
50	" "	0	+	0	*B. bronchisepticus* right ear.
51	" "	0	+	0	*Staphylococcus albus* both ears.
52	" "	0	+	Serum.	" " " "
53	" "	0	+	Red drum.	*B. bronchisepticus* right ear.
54	" "	0	+	Serum.	" " and *Staphylococcus albus* right ear.
55	" "	0	+	Viscous pus.	*B. bronchisepticus* both ears.
56	" "	Died of pneumonia.			

of rabbit snuffles, it was thought advisable to determine whether intranasal instillation of this organism was followed by otitis media.

A group of fourteen rabbits from the same source as those used in the previous experiments was selected. Into the nasal passages of the animals 1 cc. of a 16 hour culture of four strains of *Bacillus bronchisepticus* was introduced. Table VI summarizes the results. One animal died of a localized abscess-like pneumonia. All of the survivors became carriers of the organism, although no symptoms of snuffles developed. At autopsy the nasal passages of all were clean and the middle ears appeared normal in all except four. In one of these the ear drum and the lining of the tympanic cavity appeared bright red, while the cavity itself contained a slight amount

TABLE VII.

Summary of Results of Experimental Otitis Media.

Experiment.	No. of rabbits.	Snuffles.		Per cent.	Carriers.	Per cent.	Otitis.	Per cent.	Cultures.
		Died.							
1/4 cc. *Bacterium lepisepticum* intranasally.....	20	3	11	65	13	76	14	82	13 *Bacterium lepisepticum.*
1 cc. *B. bronchisepticus* intranasally.....	16	3	0	0	13	100	4	30	4 *B. bronchisepticus.*
Controls.....	11	0	0	0	0	0	0	0	1 diphtheroid.

of serous fluid. In two other cases a small amount of serum was noted. Only one showed a thick viscous material, mucoid in nature, not like the pus encountered in the above experiments. *Bacillus bronchisepticus* was isolated from the nasal passages of all the rabbits and from the ears of the four mentioned above. It was obtained in pure culture from three of these cases, but in the fourth was associated with *Staphylococcus albus.*

Controls.—This entire series of experiments was controlled by selecting a group of eleven rabbits from the same stock and keeping them under the same living conditions as the experimental animals. At the end of the period of observation they were killed and autopsied. None of them had macroscopic or microscopic evidence of nasal

infection, and none showed signs of otitis media. Cultures from the nares showed no *Bacterium lepisepticum*. Those from the middle ears were found to be sterile save in the case of two animals. From these, a few diphtheroid organisms were recovered.

This experiment shows, therefore, that rabbits raised under conditions which preclude exposure to *Bacterium lepisepticum*, as to certain other infective agents, are free of middle ear infection but that this organism dropped through the external nares into the upper nasal passages of such animals is capable of inducing a purulent otitis media similar in every respect to that which occurs spontaneously in stock and experimental animals.

SUMMARY.

A common form of otitis media occurring in rabbits is described. The condition in the great majority of instances is associated with the inflammation of the upper respiratory tract known as snuffles and with the presence of *Bacterium lepisepticum*. This organism, when introduced into the external nares of rabbits hitherto protected from infection with it, is capable of inducing a purulent otitis media indistinguishable from the natural one.

In the rabbit the connection between the nasal passages and the middle ear is such that a pathogenic agent introduced into the nose may readily invade the entire group of cavities connected with the upper respiratory tract.

STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

VIII. Nature of the Oxidation-Reduction Systems in Sterile Pneumococcus Extracts.

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INTRODUCTION.

Preceding papers (1–7) have described a number of different oxidation-reduction processes which are exhibited by sterile extracts of pneumococci: the production of peroxide, the consumption of molecular oxygen, the reduction of methylene blue, the oxidation of hemotoxin, and the oxidation of hemoglobin with formation of methemoglobin, as well as the oxidation of various endocellular hydrolyzing enzymes.

From the nature of these reactions it is evident that the sterile extracts contain active oxidation-reduction systems. These active systems consist of two components: (1) a thermolabile constituent of the pneumococcus cell which is not removed by washing; (2) thermostable substances which are lacking in washed cells and which are not necessarily of pneumococcus origin, since they may be supplied by muscle infusion and yeast extract.

Two types of sterile pneumococcus extracts have been prepared: (1) broth extracts of unwashed cells, (2) phosphate solution extracts of washed cells. The first type of extract contains both "complete" and active systems for peroxide production, oxygen consumption, hemoglobin oxidation, and reduction of methylene blue and certain other substances. The second type of extract contains only "incomplete" systems which initiate the oxidation-reduction processes only upon the "completion" of the systems by the addition of meat infusion, yeast extract, or certain other substances. The essential difference, then, between the "complete" systems in broth extracts of unwashed cells and the "incomplete" systems in saline extracts of washed cells is the presence or absence of the thermostable constituent; *i.e.*, of autoxidizable or easily oxidized substances. The

distinctions between the two types of pneumococcus extract are graphically presented in Table I.

The activities of the extract which have been reported previously may be grouped under two headings, oxidations and reductions. All of the oxidations reported are examples of "oxygen activation" and represent oxidations of substances themselves not reactive with molecular oxygen. In the absence of oxygen these same sterile extracts manifest strong reducing powers—a characteristic common to all living cells. Thus, the sterile cell extracts exhibit two general functions: in the presence of molecular oxygen, the formation of oxidizing agents, or "oxygen activation;" in the absence of molecular oxygen, the establishment of reducing conditions which are manifested in the reduction of such substances as methylene blue and methemoglobin. Moreover, not only are the two types of reactions induced by the same extract, but the active systems themselves which in the presence of molecular oxygen form the oxidizing agents are apparently identical or at least closely related to the systems which establish the reducing conditions in the absence of oxygen (2, 4).[1] These relations may be

TABLE I.

Oxidation-Reduction Systems of Sterile Extracts of Pneumococcus.

Sterile extract of.	Oxidation-reduction systems.	Components of oxidation-reduction systems.		Activity.						
				Oxidation. "Oxygen activation."				*Reduction.*		
		Thermolabile substances (*C**).	Thermostable substances (*RH*†).	Peroxide formation.	Hemoglobin oxidation.	Oxidation of hemotoxin.	Oxidation of enzymes.	Consumption of molecular oxygen.	Reduction of methylene blue.	Reduction of methemoglobin.
Unwashed pneumococcus cells.	"Complete."	+	+	+				+		
Washed pneumococcus cells.	"Incomplete."	+	−	−				−		

* *C* = thermolabile substance of bacterial origin; not removed by washing cells.

† *RH* = thermostable substance removed by washing bacteria; present in heated extracts of yeast, of animal and plant tissues.

[1] In an earlier paper (2) it was suggested that the essential difference between peroxide production and methylene blue reduction consisted in the nature of the hydrogen acceptor or oxygen donator. However, it now seems preferable of interpret the reduction activities of the extract upon the basis of the conception outlined by Clark (8), rather than upon the basis of oxygen or hydrogen transport.

presented schematically as follows, using the symbols *C* to represent the thermolabile cellular constituent and *RH*, the thermostable constituent of the active systems.

1. Pneumococcus extracts in the presence of molecular oxygen.

   $C + RH + O_2 \rightarrow$ oxidizing agents.
   Oxidizing agents + Hb→MetHb.
   " " + hemotoxin or sucrase→inactive hemotoxin or inactive sucrase.

2. Pneumococcus extracts in the absence of molecular oxygen.

   $C + RH \rightarrow$ reducing conditions under which methylene blue and methemoglobin are reduced.

If the active system be considered from this view-point, the inactivation of the extract by exposure to air might be assigned to any one of several different causes. If the thermostable constituents represented by *RH* were irreversibly oxidized, the inactivation of the system would be a natural sequence of the oxidation of the extract. However, the oxidation of the extract may not result in the exhaustion of *RH* substances, either because of the presence of an excess of these substances or because they represent, at least in part, molecules which (in the presence of *C*) may be alternately oxidized and reduced. If this is the case, the inactivation of the extract would seem to be due to the destruction or inactivation of the thermolabile cellular substance which has been designated *C*. It has been shown in preceding papers that other labile substances such as the intracellular enzymes and the endohemotoxin which are contained in these same extracts, although themselves not directly involved in the oxidation processes, are in turn destroyed by the oxidizing agents which are formed when the "complete system" type of pneumococcus extract is exposed to air. Hence the thermolabile cellular component of the oxidation system, although not actually oxidized in the initial reaction, may be inactivated by the oxidizing agents which accumulate in the extract by reactions analogous to those which under similar conditions inactivate sucrase and hemotoxin.

As indicated above, the inactivation of the described oxidation-reduction systems of the sterile pneumococcus extracts may be due to the destrcution or exhaustion of either of the two proposed constituents of these systems. The following experiments are designed to determine which of these constituents is inactivated during the oxidation of pneumococcus extracts. The experiments consist in large part of attempts to "reactivate" extracts which have been inactivated through oxidation, by adding to them fresh meat infusion (*RH*) or unoxidized washed cell extract (*C*). In addition, the relative ease

of oxidation of the cellular component of the oxidation systems is compared with the susceptibility to oxidation of other labile substances of pneumococcus origin, such as the hemotoxin and the hydrolyzing enzymes contained in these extracts. The investigation has been confined to the methylene blue-reducing, and hemoglobin-oxidizing systems, as type examples of the reducing and oxidizing activities of the sterile extract.

EXPERIMENTAL.

Methods.

The sterile pneumococcus extracts used in this investigation have been prepared in exactly the same manner described in a previous paper (7). Both types of extract have been used. To emphasize the fact that only the broth extract of unwashed cells contains the "complete" oxidation-reduction systems, this type of extract is frequently referred to in the text as the "complete system" type of extract. For a similar reason, the phosphate solution extract of washed cells is termed "incomplete system" type of extract. As in previous papers, the term "reduced" extract applies to extracts which have been protected from oxidation; extracts which have been exposed to air are termed "oxidized" extracts. The sterility of all preparations has been carefully controlled.

The procedure employed in tests of methylene blue reduction, hemoglobin destruction, hemolysis, as well as the tests of activity of the hydrolyzing enzymes, was the same as that previously described (3–6). The meat infusion used in this study is also described in a former paper (2).

Inactivation of Pneumococcus Oxidation-Reduction Systems in Unwashed Cell Extracts Exposed to the Air.

Sterile broth extracts of unwashed pneumococci contain the "complete" oxidation-reduction systems. When this type of extract is exposed to the air, marked oxidative reactions occur, such as peroxide production, hemoglobin oxidation, and oxygen consumption. One of the results of these oxidation processes is the complete inactivation of the systems responsible for the oxidation-reduction reactions. If stored in the reduced state protected from molecular oxygen, these extracts retain their activity for many months even at room temperature. These facts may be recalled from previous reports, which it is unnecessary to review in detail.

Attempts to "Activate" Extracts Which Have Been Rendered Inactive by Exposure to Air.

1. Failure of Activation of Oxidized Extracts by the Addition of the Thermostable Component (RH).

If the inactivation of extracts which have been exposed to air is due to the irreversible oxidation of the thermostable component (*RH*) (*i.e.* to the exhaustion of easily oxidized molecules), the addition of meat infusion to the oxidized extract should result in its activation in

TABLE II.

Effect of the Addition of the Thermostable Component to Pneumococcus Extracts Which Have Been Inactivated by Exposure to Air.

Broth extract of unwashed cells which had been inactivated by exposure to air.		Reduced broth extracts of unwashed cells (thermostable component).		Meat infusion (thermostable component).	PO_4 solution.	Methylene blue (1:2,000) or HbO_2 (2.0 mg).	Methylene blue reduction.	Hemoglobin oxidation (formation of methemoglobin).	
Unheated.	Heated.	Unheated.	Heated.					5 min.	8 hrs.
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*			
		0.4			0.4	0.2	Complete, 2 hrs., 30 min.	+	+
0.8						0.2	Partial, 3 days.	—	—
0.4			0.4			0.2	" 3 "	—	—
0.4				0.4		0.2	" 3 "	—	—
	0.4		0.4			0.2	" 3 "	—	—
	0.4		0.4	0.4		0.2	" 3 "	—	—

the same manner as does the addition of meat infusion to the washed cell extracts (2, 3, 5).

Similarly, the addition of an extract of unwashed cells in which the thermolabile component has been destroyed by heat should have the same effect as the addition of meat infusion, since both contain only the thermostable substances. If it is the thermostable substances which are destroyed as a result of the oxidation, the addition of fresh substances of a similar nature should restore the activity of the oxidized extract. A number of experiments have been made to test these relations; a typical example is given below.

Broth extract of unwashed cells ("complete system" type of extract) was exposed to air for 18 hours at 37°C. The extract upon aeration formed peroxide, but after 18 hours the peroxide had practically disappeared. As shown in previous experiments, the extract after oxidation no longer contains "complete" or active systems for methylene blue reduction and hemoglobin destruction. The thermostable component of the oxidation systems (as represented by fresh meat infusion and by heated "reduced" broth extract) was added to the oxidized extract and tests made of the methylene blue-reducing and hemoglobin-oxidizing powers of the mixtures.

Results of experiments of this nature (Table II) prove that the activity of cell extracts which has been destroyed through oxidation cannot be restored by the addition of the thermostable components present in meat infusion or heated extracts. Therefore the exhaustion of the thermostable component cannot explain the inactivation of these extracts by oxidation.

2. Activation of Oxidized Extracts by the Addition of the Thermolabile, Cellular Component.

The failure to "reactivate" oxidized extracts by the addition of the thermostable component of the oxidation systems indicates that the thermolabile, cellular constituent of the system is injured by the oxidation processes. Provided the thermolabile component alone is destroyed, the oxidized extract will still contain the thermostable component. To investigate this question, experiments have been conducted in which washed cell extracts, which have been shown to contain only the thermolabile component, have been added to extracts previously inactivated by oxidation. The experiments, therefore, are tests of the possibility of "reactivating" the oxidized, inactive systems by the addition of the thermolabile, cellular component.

Thermolabile Component.—The thermolabile, cellular component was obtained by the use of an extract of washed cells which had previously been found to contain only the thermolabile component and to be by itself inactive.

Thermostable Component.—The thermostable component was obtained by destroying the thermolabile component by heating and by oxidation. As controls, both the oxidized and unoxidized extracts were used in the heated state.

Test Mixtures.—Mixtures were made of known amounts of the thermolabile and thermostable component and the activity of the resultant mixture was determined by observations of the methylene blue-reducing and hemoglobin-oxidizing powers. The results are given in Table III.

In the previous experiment it was shown that the addition of the thermostable component to an oxidized extract does not restore its activity. In the present experiment, however, it is evident that the addition of the thermolabile component to the oxidized extract results in the complete restoration of activity. Hence, the loss in activity of an oxidized extract represents the inactivation of the thermolabile component. On the other hand, the thermostable component

TABLE III.

Effect of the Addition of the Thermolabile Component to Pneumococcus Extracts Which Have Been Inactivated by Exposure to Air.

PO_4 solution extract of washed cells (thermolabile component).		Broth extract of unwashed cells previously inactivated by exposure to air.		"Reduced" broth extract of unwashed cells (thermostable component).	Meat infusion (thermostable component.)	Methylene blue reduction.	MetHb formation.	
Unheated.	Heated.	Unheated.	Heated.	Heated.			30 min.	8 hrs.
cc.	cc.	cc.	cc.	cc.	cc.			
	0.4	0.4				Incomplete, 3 days.	—	—
0.4		0.4				Complete, 3 hrs., 15 min.	+	+
0.4			0.4			" 3 " 15 "	+	+
0.4				0.4		" 3 "	+	+
0.4					0.4	" 3 " 15 min.	+	+
	0.4				0.4	Incomplete, 3 days.	—	—

remains relatively unaffected not only by oxidation but by heat, since in the present experiment, the heated and unheated oxidized extracts exhibit comparable activity when suitably complemented by the thermolabile component. It is interesting to note that the thermostable component is but little if at all affected by the oxidation of the extract. In the presence of the thermolabile constituents, the thermostable substances in oxidized extracts function practically as well as do those present in extracts which have been heated but never exposed to oxi-

dation. Although not quantitative, these data suggest that the thermostable constituents are either present in great excess, or that they consist, in part, of substances which may be alternately oxidized and reduced.

Effect of Exposure to Air upon the Subsequent Activity of Washed Cell Extracts.

The preceding experiments have demonstrated that it is the thermolabile component (an actual constituent of the pneumococcus cell) which is destroyed during the oxidation of the "complete system" type of extract. In previous studies it has been found that during the oxidation of these extracts other thermolabile substances of cellular origin, such as pneumococcus hemotoxin, sucrase, amylase, raffinase, and inulase, are also destroyed. In spite of the destruction of the hemotoxin and enzymes in oxidized unwashed cell extracts, in the "incomplete" or washed cell extracts these substances proved relatively stable when exposed to air. The following experiment was conducted to determine the effect of exposure to air upon the subsequent activity of the thermolabile cellular component of the oxidation-reduction systems in washed cell extracts.

Phosphate solution extract of washed pneumococci (the "incomplete system" type of extract) was exposed to air in a shallow layer in an Erlenmeyer flask, held in a water bath at 37°C. After 1 hour, 3 hours, and 24 hours exposure, 0.6 cc. of the aerated extract was placed in duplicate reduction tubes together with 0.6 cc. of meat infusion or 0.6 cc. of yeast extract, and 0.2 cc. of 1:2,000 methylene blue was added to each tube. The reduction tubes were sealed and incubated at 37°C. The time required for dye reduction by the extract previously exposed to air was compared with that required by the extract which had not been exposed to air.

The effect of exposure to air upon the subsequent hemoglobin-oxidizing power was tested by the usual method. The results are given in Table IV.

The results of experiments of this type (Table IV) indicate that when exposed to air in the washed cell extract, the thermolabile constituent of the methylene blue-reducing and hemoglobin-oxidizing systems suffers a gradual loss in activity. This loss is extremely slow, and demonstrable only after prolonged periods of exposure. This is in striking contrast to the rapid inactivation which occurs

whenever the "complete system" type of extract is exposed to air. However, the fact that the potential activity of this thermolabile cellular substance is injured at all under these conditions is evidence of greater susceptibility to oxidation than that exhibited by the enzymes and hemotoxin in the same extracts. Although both are extremely labile substances, neither pneumococcus hemotoxin nor sucrase is inactivated to a detectable degree when washed cell extracts are exposed to molecular oxygen for 24 to 48 hours. Since it is gradually destroyed by prolonged exposure to air in the washed cell extracts, the thermolabile component of pneumococcus oxidation-

TABLE IV.

The Effect of Exposure to Air upon the Subsequent Activity of Washed Cell Extracts.

Washed cell extract exposed to air at 37°C. for.	Meat infusion added to extract after exposure to air.	Activity of washed cell extract after exposure to air.			
		Methylene blue reduction (time for reduction).	Hemoglobin oxidation (methemoglobin formation) after following periods at 37°C.		
			30 min	1 hr.	4 hrs.
hrs.					
0		2 hrs., 30 min.	+	++	++
1		2 " 30 "	+	++	++
3		2 " 30 "	+	++	++
24		3 " 15 "	−	+	++

reduction systems appears to be the most easily oxidized among all of the labile constituents of the pneumococcus cell which have yet been described.

Comparison of the Influence of Heat and of Oxidation upon the Subsequent Activity of the Various Labile Cellular Substances in Sterile Extracts of Washed Pneumococci.

The results of a previous investigation showed that the known endocellular enzymes of Pneumococcus vary in their relative resistance to oxidation, their resistance decreasing in the following order: peptonase, lipase, bacteriolytic enzyme, sucrase, and other carbohydrate-hydrolyzing enzymes. This order of relative resistance is maintained whether the oxidizing agents employed be the reagent hydrogen peroxide or the oxidizing agents formed during the oxidation of pneumo-

coccus extracts. It was further found that the order of resistance of these enzymes to oxidation agrees with the relative order of resistance of the same enzymes to heat. Data previously presented which are collected together in the following pages, demonstrate that this relation does not apply to the thermolabile cellular constituent which is concerned in methylene blue reduction and hemoglobin oxidation.

TABLE V.

Comparison of the Influence of Heat and of Oxidation upon the Subsequent Activity of the Various Cellular Substances in Sterile Pneumococcus Extracts.

Labile constituent of pneumococcus cell.	Resistance to heat.	Resistance to oxidation.	
		Exposed to the oxidation products which are formed when unwashed cell extracts are exposed to air.	Exposed 24 hrs. to 0.003 per cent H_2O_2.
Labile cellular constituent of methylene blue-reducing and hemoglobin-oxidizing systems.	Completely destroyed. 10 min at 65°C.	Completely destroyed.	Completely destroyed.
Lipase.	Completely destroyed. 10 min. at 65°C.	Unaffected.	Unaffected.
Sucrase.	Completely destroyed. 10 min. at 55°C.	Partially destroyed.	Partially destroyed.
Hemotoxin.	Completely destroyed. 1 min. at 55°C.*	Completely destroyed.	Partially destroyed.

* Unpublished observation.

The oxidized portion of extract was prepared as follows: A titrated dilute solution of "Dioxogen" was added to washed cell extract in amount sufficient to give a concentration of 0.003 per cent of hydrogen peroxide. The peroxide-treated extract was sealed with vaseline and gave a faint peroxide test with benzidine. The heated extract was exposed to 55°C. for 10 minutes in a sealed tube.

The activity of the various cellular constituents in portions of the original extract, heated extract, and peroxide-treated extract was tested and described in a preceding paper (6).

The results of the above experiments, together with data from preceding papers, are summarized in Table V. It is evident (Table V)

that the thermolabile cellular constituent which is involved in the described oxidation reactions is extremely sensitive to hydrogen peroxide, an oxidizing agent similar in nature to those which accumulate in aerated pneumococcus extracts. In a preceding paper (6) a study was made of the resistance of different enzymes of Pneumococcus to various concentrations of hydrogen peroxide. The resistance of the different enzymes to this oxidizing agent was found to be comparable to the relative resistance of the same enzymes to heat; *i.e.*, enzymes which proved more resistant to heat were also more resistant to hydrogen peroxide. Quite different relations are evident in the above results as concerned the labile cellular component of the oxidation-reduction system. This labile constituent of Pneumococcus is far more sensitive to oxidation than is the pneumococcus lipase although both exhibit approximately equal heat resistance. Further, hemotoxin and sucrase, although much more heat-labile than the cellular constituent of the oxidation systems, are less susceptible to inactivation by oxidation.

Destruction of the Thermolabile Constituent of the Oxidizing-Reducing Systems in Aerobic Cultures of Pneumococcus.

The destruction of hydrolyzing enzymes which occurs during the oxidation of sterile pneumococcus cell extracts is reflected in differences in the enzyme activity of the filtrates of anaerobic and aerobic cultures (7). The endocellular substances freed by autolysis are destroyed by oxidation in aerobic cultures in a manner apparently analogous to the destruction of the same substances when exposed to the oxidation products formed in the sterile cell extracts. In another paper from this laboratory (9) it has been shown that the methemoglobin-forming system is inactivated in aerobic cultures just as in "oxidized" cell extracts. The rapid destruction of both the methylene blue-reducing and hemoglobin-oxidizing systems when the cell extracts are oxidized has been shown in the present paper to be due to the inactivation of the thermolabile cellular constituent. Experiments were made to determine if the destruction of this constituent is also the cause of the inactivation of the systems in the case of aerobic culture filtrates. Since the cellular component of the oxidation-

reduction system is endocellular, the tests were made on autolyzed cultures.

It was found that filtrates of autolyzed anaerobic cultures possess an active methylene blue-reducing system, as well as the hemoglobin-oxidizing system reported previously, while filtrates of aerobic cultures do not possess these active systems. The inactivity of the filtrates of the aerobic cultures is due to the destruction of the thermolabile cellular substances, probably by the same reactions as those which inactivate the sterile cell extracts.

DISCUSSION.

In the preceding papers of this series (1–7), the oxidizing and reducing activities of sterile pneumococcus extracts have been described. The nature of these reactions is determined by the presence or absence of molecular oxygen. In the presence of molecular oxygen the active extracts have been shown to exhibit the following activities: consumption of molecular oxygen, formation of peroxide, oxidation of hemoglobin, oxidation or destruction of the endohemotoxin and intracellular enzymes contained in pneumococcus extracts. All of these oxidations are examples of "oxygen activation" whereby substances themselves not reactive with molecular oxygen are easily oxidized by agents formed during the oxidation of other substances. If molecular oxygen is absent or the supply restricted, these same extracts establish conditions which result in the reduction of methylene blue and methemoglobin. Analysis of these processes shows that the active systems consist of two components, one of which is thermolabile, the other thermostable. The thermolabile component is an actual constituent of the Pneumococcus and is not removed by washing the cells. The thermostable component is not necessarily of pneumococcus origin and may be supplied by adding yeast extract or meat infusion to extracts prepared from washed bacteria. The marked oxidation reactions which occur when an active extract is exposed to air, result in the inactivation of the oxidizing and reducing systems. The results of the present study show that the thermostable substances are still active in oxidized extracts and that the inactivation of the oxidation-reduction systems is due to the destruction of the thermolabile cellular constituent. This cellular substance is not only rapidly de-

stroyed by products formed during oxidation but is gradually inactivated by molecular oxygen itself. Moreover, the labile component of the oxidation system is more susceptible to the action of the reagent hydrogen peroxide than is any other known intracellular substance of pneumococcus origin.

In the interpretation of the interaction of these two components with molecular oxygen, the formula $C + RH$ has been used to represent the system in these extracts by which peroxide is formed and hemoglobin oxidized. It is assumed that the thermostable substances (RH) are the actual source of the oxidizing agents and that the thermolabile cellular substance (C) serves as a catalyst in accelerating the reactions involved. The fact that the cellular component is inactivated by oxidation is not a serious objection to its catalytic nature. Evidence presented in another paper makes it clear that thermolabile cellular substances of ferment nature, such as the hydrolyzing enzymes, which are not concerned in oxidation and reduction, are inactivated by agents formed during the oxidation of pneumococcus extracts. In view of the high degree of susceptibility to peroxides of the thermolabile component, it is reasonable to assume that the oxidation-reduction systems may be destroyed by the same reactions which inactivate the hemotoxin and enzymes contained in the extracts. In fact, the inactivation of catalytic agents by products of their own activity is a common phenomenon. The order of reactions would seem to be analogous to that previously proposed for the oxidation of hemoglobin, hemotoxin, and sucrase.

(1) $C + RH + O_2 \rightarrow$ oxidizing agent.
(2) Oxidizing agent $+ C \rightarrow$ inactive C.

Here it is inferred that C functions in equation (1) as a thermolabile, cellular catalyst. Its inactivation in equation (2) is assumed to represent simply the destruction of a labile substance which is very easily inactivated by the oxidizing agents present in the aerated extract.

While our knowledge of the oxidation-reduction systems of Pneumococcus is still incomplete, the work thus far accomplished has resolved the system into two components, the nature of which may be characterized as follows: The component RH represents substances which are not present in the Pneumococcus after thorough washing. These

substances are relatively heat-stable, resisting boiling for prolonged periods of time. They are present in water or alcohol extracts of muscle, yeast, and vegetable tissue. By themselves, these substances react slowly with molecular oxygen to form oxidizing agents, and, in the absence of molecular oxygen, they establish conditions under which methylene blue and methemoglobin are slowly reduced. Although separately these substances are but slowly reactive, in the presence of the second component the reactions of oxidation and reduction are markedly accelerated. This labile cellular component, referred to as *C*, represents substances which are resident in the Pneumococcus and are not removed by washing the cells. This component is thermolabile, being inactivated by exposure to 65°C. for 10 minutes. By itself it is wholly non-reactive with molecular oxygen and possesses no reducing power. This cellular component seems to be catalytic in nature, since it greatly accelerates reactions of oxidation and reduction in the presence of the other component of the system. When present together, these substances constitute systems responsible for many of the biological oxidations and reductions of the living cell.

SUMMARY.

1. The systems responsible for oxidation and reduction in the pneumococcus cell have been shown to consist of two components, the nature of which has been discussed.

2. The oxidizing and reducing power of active extracts of pneumococci is inactivated by exposure to air. This loss in activity is due to an inactivation of the thermolabile cellular component consequent on secondary reactions analogous to those which under similar conditions destroy the hemotoxin and enzymes in pneumococcus extracts.

BIBLIOGRAPHY.

1. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 347.
2. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 543.
4. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 745.
5 Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xxxix, 757.
6. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 405.
7. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 423.
8. Clark, W. M., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 443.
9. Morgan, H. J., and Neill, J. M., *J. Exp. Med.*, 1924, xl, 269.

STUDIES ON THE OXIDATION-REDUCTION OF HEMOGLOBIN AND METHEMOGLOBIN.

I. The Changes Induced by Pneumococci and by Sterile Animal Tissue.

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Certain principles governing the operation of the oxidation-reduction systems of Pneumococcus have been established in previous papers from this laboratory (13, 3, 4, 67). These studies are extended in the present paper by an investigation of the oxidation-reduction activities of Pneumococcus based upon the physical-chemical relations between oxyhemoglobin, hemoglobin, and methemoglobin. The two general types of oxidizing and reducing activity of Pneumococcus are illustrated by the action of the living cells and of sterile cell extracts upon hemoglobin and upon methemoglobin, its oxidation product. In view of the possible importance of the action of the animal tissues upon the blood pigments during pneumonia, experiments devised to test the methemoglobin-reducing activity of sterile animal tissues are also included.

HISTORICAL.

The earlier authorities (1, 41, 42, 58, 92) presented considerable evidence for the belief that hemoglobin is the reduction product of methemoglobin. The hemoglobin formed by the action of reducing agents upon methemoglobin was identified spectroscopically, and it was further observed that oxyhemoglobin or carbon monoxide hemoglobin did not appear until oxygen or carbon monoxide was added to the reduction product of methemoglobin. However, in consequence of the overemphasis upon oxygen in oxidation, the true interrelations of these blood pigments were not perceived. Küster (56), however, expressed the opinion that the iron of the hemoglobin radical which possessed two valencies (ferro) had three valencies (ferri) in the methemoglobin molecule. For the controversial development of present knowledge of the relations of these blood pigments the reader

may be referred to the following authorities (36, 37, 38, 42, 48, 49, 78, 79, 90, 84, 85, 86, 72, 24, 58, 41, 46, 47, 69, 7, 17, 45, 28, 56, 74, 44).

The interrelation of hemoglobin, oxyhemoglobin, and methemoglobin has now been clarified by Conant's (14) electrochemical study in which it is proved that the oxidation-reduction potential in a mixture of the three blood pigments is referable to the potential between hemoglobin and methemoglobin. Thus, hemoglobin is the reduction product of methemoglobin and methemoglobin is the oxidation product of hemoglobin. Since the transformation of hemoglobin to methemoglobin involves the loss of one electron, Conant believes the oxidation of hemoglobin to consist in the change of ferrous iron in hemoglobin to the ferric iron in methemoglobin.

The Biological Reduction of Methemoglobin to Hemoglobin.—That bacteria can reduce methemoglobin to hemoglobin was reported in 1895 by Filipowski (22), and later by Grüter (27) and Schnabel (80). However, similar observations were made by Hoppe-Seyler (37) in 1878 and by Araki (1) in 1890, who report the transformation of methemoglobin to hemoglobin "*durch Fäulnis*" and that the hemoglobin thus formed can be converted to oxyhemoglobin or to carbon monoxide hemoglobin by admitting the appropriate gas.

Reports that the methemoglobin is reduced to hemoglobin by animal tissues appear in the early literature (33, 34, 61, 22, 17, 52); but only Schnabel demonstrated it experimentally. The reducing action has been ascribed to different tissues (33, 34, 52–55, 9). Many investigators suggest the liver as most active in this respect.

Accumulation of Methemoglobin in the Blood of Animals Injected with Methemoglobin-Forming Chemicals.—It is well known that many chemicals induce the formation of methemoglobin when introduced into the body (21, 17, 16, 55, 93). Some which are inactive *in vitro* are reported to cause the formation of the pigment *in vivo* (19, 44). More frequently, chemicals which cause marked methemoglobin formation *in vitro* may be injected without the accumulation of it in detectable amounts. The animal body evidently offers considerable resistance to its accumulation. A number of the early workers (33, 34) finding that methemoglobin rapidly disappears from the blood concluded that it is reduced to hemoglobin by the tissues. Later Haldane, Makgill, and Mavrogordato (1897) gave rabbits sublethal doses of nitrites. In a typical instance, over half of the blood pigment had become methemoglobin within an hour, and the rabbit showed marked symptoms of oxygen want; but after 3½ hours, the methemoglobin was reduced to 6 per cent and the rabbit appeared normal. Lewin's (59) and Dennig's (16) findings are of similar nature. Haldane's contribution may be considered sufficient evidence that the animal body, if not overtaxed, can reduce the methemoglobin which is formed in the blood cells by the injection of certain chemicals. It is probable that there is considerable justification for the earlier emphasis of Hayem (33, 34), Dittrich (17), and others on the importance of the persistence of the methemoglobin inside of the red cell as a factor in favor of the reduction to hemoglobin.

The Occurrence of Methemoglobin in the Blood in Pneumonia and Other Infections Due to Methemoglobin-Forming Bacteria.—Following the observation of methemoglobin formation by Pneumococcus *in vitro*, cyanosis in pneumonia was frequently attributed to the presence of methemoglobin. However, it has been shown that the usual cause of the cyanosis is not the presence of methemoglobin but that it is due to an increased percentage of reduced hemoglobin in the capillaries (70, 87, 60). In spite of the marked formation of methemoglobin by Pneumococcus, this substance has rarely been demonstrated in the blood of pneumonia patients. In the blood of laboratory animals with a profound pneumococcus septicemia, significant amounts of methemoglobin have been observed (70, 88). Kämmerer (50) has also reported the presence of methemoglobin in "rusty" sputum from pneumonia patients. A marked decrease in the "oxygen capacity" of the blood apparently occurs only in the most severe pneumonias (87, 31, 39). This fact indicates that if a significant amount of methemoglobin is formed in less severe pneumonias, it must be reduced to hemoglobin by the tissues. Medigreceanu (64), working in this laboratory, found that the indophenol oxidase activity of the tissues of pneumococcus-infected rabbits is markedly less than that of the tissues of normal animals. Stadie (88) has shown that in severely infected animals,—in which more methemoglobin is produced than can be reduced by the tissues of the diseased animal,—a part of the methemoglobin formed is eliminated from the blood stream or destroyed to other products as shown by a drop in the "total pigment."[1]

Much the same relations are evidenced in a survey of the literature on the accumulation of methemoglobin in the blood of animals suffering from infections with other methemoglobin-forming bacteria. Emmerich and Tsuboi (20) reported the presence of methemoglobin in the blood of animals infected with Asiatic cholera, although other workers have failed to detect this pigment under apparently similar conditions. An interesting exception to the general rule is the observation of Boycott (9) on the presence of large amounts of methemoglobin in the blood of a number of rats infected with Gaertner's bacillus. He reports the most marked accumulation of methemoglobin *in vivo* which has appeared in bacteriological literature; the microbic agent, which, interestingly enough, is not a marked methemoglobin former *in vitro*, caused a severe injury to the liver, probably the most active reducing tissue of the body. These relations are of especial

[1] There is considerable evidence for the belief that the destruction of hemoglobin which occurs in the normal organism proceeds by way of methemoglobin, which if not reduced to hemoglobin, is destroyed to globin and hematin ($HbO_2 - O_2 \rightarrow Hb - e \rightarrow MetHb \rightarrow$ globin and hematin), methemoglobin formation, in fact, being a preliminary stage in the breaking down of hemoglobin to hematin and globin. Thus, methemoglobin free in the plasma, if not reduced by the tissues to hemoglobin, would be started on the course supposedly followed in the normal destruction of blood pigments. Evidence that under certain conditions methemoglobin is less stable than hemoglobin is presented in a later paper.

interest from the standpoint of a balance between the forces producing methemoglobin and those responsible for its reduction in the tissues.

The Oxidation of Hemoglobin to Methemoglobin by Bacteria.—The identification of methemoglobin as a product of the action of Pneumococcus and other bacteria upon blood, and an understanding of the mechanism of its formation has been only gradually accomplished (25, 77, 83, 73, 8, 27, 71, 13, 80, 6, 50, 88, 2, 62, 63, 3, 4, 67, 65, 68). Conant (14) interprets methemoglobin formation by the ferricyanide reaction as an oxidation of oxygen-dissociated hemoglobin. Evidence that this is also the case in methemoglobin formation by Pneumococcus appears in a previous paper (67).

Methods.

Bacteriological.—

Sterile Extracts of Pneumococcus.—The sterile pneumococcus extracts were prepared as described in previous papers. All were filtered and proven sterile by rigorous cultural and animal tests.

Chemical.—

The oxyhemoglobin solutions used in most of the experiments were prepared without crystallization from the well washed cells of sterile, defibrinated horse or rabbit blood. The cells were laked in sterile distilled water and were frozen and thawed several times. Sterile phosphate solution (pH 7.5) was added in amount sufficient to give a final phosphate concentration of 20 mM. These solutions were centrifuged to remove the precipitate which was formed on the addition of phosphate to the rabbit cells. In certain tests, solutions of crystalline oxyhemoglobin prepared by Heidelberger's (35) method were used.

The measurements of "oxygen content," and the determination of hemoglobin by "oxygen capacity" and "carbon monoxide capacity" were made by methods described by Van Slyke and Neill (91) with the modifications for carbon monoxide analyses described by Harington and Van Slyke (40). "Total pigment" (HbO_2 + Hb + MetHb) measurements were made by Stadie's (89) colorimetric method. All of the blood pigment determinations are reported in terms of millimols per liter.

The detections of peroxide were made by the technique described in a previous paper (3) with the exception that the alcohol precipitate of potato juice (23) was used as "peroxidase" in the place of the whole potato tissue previously employed.

The Reduction of Methemoglobin by Living Pneumococci and by Sterile Pneumococcus Extracts.

In previous papers, it has been shown that methylene blue is promptly reduced by sterile extracts of pneumococci. From the conception of oxidation and reduc-

tion outlined by Clark (11) the reduction of methylene blue by living pneumococci or by sterile pneumococcus extracts may be interpreted as an index of the establishment of a reduction potential at which methylene blue is transformed to methylene white (10, 12). Since Conant (14) has shown that hemoglobin is the reduction product of methemoglobin, the relation of methemoglobin to hemoglobin in this respect is similar to that of methylene blue to methylene white. From the "intensity" standpoint, therefore, provided that a sufficiently high reduction potential is established by the living cells or the sterile extract, methemoglobin will be reduced to hemoglobin just as methylene blue is reduced to methylene white.

TABLE I.

Reduction of Methemoglobin by Living Pneumococci and by Sterile Pneumococcus Extract.

Reaction mixture.		Total pigment (HbO_2 + Hb + MetHb).	O_2 content (HbO_2 + O_2).	HbO_2*	HbO_2 + Hb	MetHb	MetHb reduced to Hb.
		mM	mM	mM	mM	mM	mM
5 cc. broth suspension of pneumococci + 15 cc. solution of MetHb + Hb.	Original.	4.50	1.00	0.74	0.74	3.76	
	After 6 hrs. at 37°C., in a sealed system.	4.50	0.00	0.00	3.25†	1.25	2.51
3 cc. sterile pneumococcus extract + solution of MetHb + Hb.	Original.	2.95	0.76	0.50	0.50	2.45	
	After 15 hrs. at 37°C., in a sealed system.	2.95	0.00	0.00	2.60‡	0.35	2.10

* Corrected for dissolved O_2.

† Determined by O_2 capacity.

‡ Determined by CO capacity.

A survey of the literature reveals the early suggestions that living bacteria may reduce methemoglobin to hemoglobin. Experiments were conducted to furnish quantitative confirmation of these suggestions and to show whether sterile extracts of pneumococcus cells can induce the reaction. Suspensions of living cells and relatively large amounts of sterile pneumococcus extract respectively were added to sterile solutions of methemoglobin. The systems were then sealed from air.

In these experiments "oxygen content" analyses prove the complete absence of both oxyhemoglobin and molecular oxygen. The

reduction product, hemoglobin, was determined by converting it to oxyhemoglobin and carbon monoxide hemoglobin. Typical protocols illustrating the results are presented in Table I. They show that in the absence of air, living pneumococci, and sterile cell extracts prepared from them, reduce methemoglobin to hemoglobin.

Hemoglobin Oxidation and Methemoglobin Reduction as Functions of Identical or Closely Related Systems.

That the oxidation of hemoglobin and the reduction of methemoglobin are functions of the same or closely allied systems in sterile pneumococcus extracts is indicated by the fact that conditions which influence one of these activities affect the other similarly. The susceptibility to heat is apparently the same in both instances for an extract heated 10 minutes at 65°C. neither forms peroxide nor reduces methylene blue. Extracts prepared from washed cells which are themselves inactive as regards peroxide formation and methylene blue reduction may be "activated" by the addition of certain substances such as meat infusion. In like manner, the extracts may be rendered capable of inducing hemoglobin oxidation and methemoglobin reduction. The similarity in thermolability and "activation," as in the case of other functions of these extracts, indicates that the oxidation and reduction of hemoglobin and methemoglobin are functions of like systems, the reaction induced being referable merely to the presence or absence of molecular oxygen.

As shown by other workers, washed living bacterial cells also require the presence of certain substances, such as cell washings, meat extract, and glucose, for the establishment of reducing conditions (Harden and Zilva (30); Gillespie (26) with colon bacilli) and for the formation of oxidizing agents (Cole (13) with pneumococci). It has been found in the present investigation that the principles previously established for the oxidation-reduction systems in sterile extracts of the cellular substances also apply to the oxidation-reduction systems of living, intact pneumococci. Thus, upon the addition of meat infusion, or alcoholic extracts of meat infusion or of potato juice, both the washed intact bacterial cells and the sterile extracts prepared from them manifest the ability to form peroxides and to oxidize hemoglobin as well as to reduce methylene blue and methemoglobin. Curiously enough,

however, the sterile extracts prepared by freezing and thawing washed cells do not respond to the addition of glucose, although this substance serves to "complete" the systems in suspensions of washed living pneumococci. Apparently the mechanism by which the intact living cell in the presence of glucose forms oxidizing agents or establishes reducing conditions is dependent upon the preservation of the viability and morphological integrity of the cell. The fact that the loss of the ability to respond to glucose in sterile cell extracts is reflected in both the hemoglobin-oxidizing and the methemoglobin-reducing functions is further evidence that identical or closely related systems are involved in these two reactions.

Reversibility of the Oxidation-Reduction of Hemoglobin and Methemoglobin by Pneumococci and by Sterile Pneumococcus Extracts.

The oxidation-reduction of hemoglobin and methemoglobin is a reversible process. Conant made his electrochemical study of hemoglobin and methemoglobin by titrations with ferricyanide as oxidizing agent and sodium hydrosulfite as reducing agent. The titrations may be reversed several times with a single specimen of hemoglobin-methemoglobin solution.

Living pneumococci and active sterile pneumococcus extracts which possess both oxidizing and reducing powers should shift the oxidation-reduction equilibrium of mixtures of hemoglobin and methemoglobin in a direction controlled by the presence or absence of molecular oxygen.

A number of experiments, such as will now be described, have been made to test the reversibility of the oxidation-reduction of hemoglobin and methemoglobin by pneumococcus cellular substances.

Experiment with Living Pneumococci.—10 cc. of a broth suspension of centrifuged pneumococci (Strain G, Type II) were added to 25 cc. of a fresh solution of oxyhemoglobin, containing no methemoglobin. The oxidations were effected by exposing the mixture of bacterial cells and hemoglobin to air in a 50 cc. tube. After oxidation of the hemoglobin to methemoglobin the tubes were sealed with a deep layer of sterile vaseline for the reduction period. The periods allowed for oxidation and for reduction are given in the protocol.

Experiment with Sterile Pneumococcus Extract.—5 cc. of sterile pneumococcus extract were added to 5 cc. of a mixture of methemoglobin and hemoglobin. The reduction and oxidation periods are given in the protocol.

After the intervals allowed for oxidation and reduction, the "oxygen content" and hemoglobin content were determined gasometrically. The results are presented in Table II.

Several interesting relations are evident in an analysis of the results of experiments such as those presented in Table II. The equilibrium of the reversible reaction, Hb − e⇄MetHb may be shifted in either direction by the action of pneumococcus cellular substances. Unlike the usual procedure in titrations of hemoglobin-methemoglobin solutions with the more common chemical reagents, either oxidation or

TABLE II.

Reversibility of the Hb⇆MetHb Reaction of Pneumococcus.
Experiment with Living Pneumococci.

Broth suspension of pneumococci + HbO_2 solution.	O_2 content (HbO_2 + O_2).	Hb	Hb + HbO_2*	MetHb†	Hb oxidized.	MetHb reduced.
	mM	mM	mM	mM	mM	mM
Original	6.14		5.88	0.00		
Oxidation (exposed to air 30 min.)	1.00		0.74	5.14	5.14	
Reduction (sealed 3 hrs.)	0.00	2.31	2.31	3.57		1.57
Oxidation (exposed to air 20 min.)	0.72		0.46	5.42	1.85	
Reduction (sealed 12 hrs.)	0.08	0.99	0.99	4.89		0.53
Oxidation (exposed to air 1 hr.)	0.11		0.11	5.77	0.88	

Experiment with Sterile Extracts of Pneumococcus.

Sterile extract of pneumococci + mixture MetHb + HbO_2.	MetHb + HbO_2 + Hb	O_2 content (HbO_2 + O_2).	Hb	Hb + HbO_2*	MetHb†	Hb oxidized.	MetHb reduced.
	mM	mM	mM	mM	mM	mM	mM
Original	2.40	0.68		0.42	1.98		
Reduction (24 hrs. under seal)	2.40	0.00	1.58	1.58	0.92		1.16
Oxidation (exposed to air 20 min.)	2.40	0.42	0.16	0.16	2.24	1.32	

* Determined by O_2 capacity.

† Estimated by difference ("total pigment" minus Hb).

reduction may be induced by the cellular agents, the direction of the change being dependent upon the presence or absence of molecular oxygen. The reversibility of the hemoglobin and methemoglobin in these mixtures is, however, lost after several periods of oxidation. This loss may conceivably be due to the inactivation of the bacterial substances and, in part, to the change of some hemoglobin molecules to products other than methemoglobin.

In a previous paper (67) it was stated that large concentrations of sterile pneumococcus extract will transform hemoglobin to products beyond methemoglobin, while the action of lower concentrations of the cellular substances is limited to methemoglobin formation. This statement was based upon tests made at atmospheric oxygen tension in dilute solutions of hemoglobin. A similar destruction of hemoglobin to products beyond methemoglobin was also observed with the requisite concentrations of reagent hydrogen peroxide. Other chemicals which cause methemoglobin formation may also transform the methemoglobin formed to other hemoglobin degradation products such as globin and hematin (32, 58, 59).

It should be observed that both living pneumococci and sterile extracts can establish reducing conditions in mixtures of oxyhemoglobin and methemoglobin which initially contain not only the usual dissolved oxygen but also a considerable reserve supply of molecular oxygen. Probably all of the molecular oxygen is consumed before the reduction of methemoglobin proceeds.

The power of Pneumococcus to oxidize hemoglobin is much more pronounced than its power to reduce methemoglobin. Much less time is required to oxidize a large amount of hemoglobin than is required to reduce a relatively small amount of methemoglobin. These relations are entirely reversed in the case of certain anaerobic bacteria, as will be shown in a following paper.

The Reduction of Methemoglobin by Sterile Animal Tissues.

The ability of tissues to reduce MetHb to Hb has been repeatedly involved in the past to explain the apparent resistance of the animal body to the accumulation of MetHb in the blood stream. In view of the probable importance of this reducing action, in the maintenance of a supply of hemoglobin in the blood stream, experiments were made to measure quantitatively the action of sterile animal tissues upon methemoglobin.

Methemoglobin Solutions.—The methemoglobin used has been obtained, in all cases, from sterile oxyhemoglobin solutions which, after long standing, had changed "spontaneously" to methemoglobin. The final methemoglobin solutions were approximately pH 7.5; sterile phosphate solution was added to give a final concentration of 20 mM phosphate. Tests have been made on methemoglobin obtained from crystalline horse hemoglobin (Heidelberger's method) and from the uncrystallized product obtained from the laked, washed cells of sterile horse blood. All the methemoglobin solutions were proved sterile by cultural methods.

Sterile Tissues.—Kidney, testicle, and liver tissues were obtained from an exsanguinated normal rabbit.

Reduction Tests.—The sterile tissues were added to the methemoglobin solutions and the mixture then sealed and incubated at 37°C. The sterility was proved in each test by subculture *after* the reduction period.

As a check on the liberation of the hemoglobin introduced with the tissues or the entrance into the tissues of the methemoglobin proper to the tests, "total pigment" measurements were made. Apparently, however, this was an unnecessary precaution as no differences were detected in the "total pigment" of controls and of the solution containing the tissues.

The methemoglobin solution used in the tests which are reported in Table III contained a small amount of oxyhemoglobin. In sealed tubes, however, the tissues soon consumed the oxygen and established reducing conditions.

TABLE III.

Reduction of Methemoglobin by Sterile Animal Tissues in Vitro.

Tissue.	Period of reduction at 37°C.	"Total pigment" (HbO_2 + Hb + MetHb).	Hb*	MetHb†	MetHb reduced to Hb.
	hrs.	*mM*	*mM*	*mM*	*mM*
Control		1.25	0.14	1.11	
Kidney	12	1.25	1.19	0.06	1.05
"	2	1.25	0.61	0.64	0.47
Testicle	3	1.25	0.88	0.37	0.64
Liver	3	1.25	1.14	0.11	1.00

* Determined by O_2 capacity.

† Estimated by difference ("total pigment" minus Hb).

The figures reported in Table III are evidence of the reduction of methemoglobin to hemoglobin by sterile animal tissues. Reduction is also effected by sterile plant tissues and by many bacteria, as will be shown in a later paper.

DISCUSSION.

Sterile extracts of pneumococci and living pneumococcus cells exhibit two groups or types of oxidation-reduction reactions. In the present paper these two functions may be illustrated by the action upon hemoglobin and methemoglobin. When molecular oxygen is excluded, the sterile extract reduces methemoglobin to hemoglobin, whereas when oxygen is present the extract forms oxidizing agents which oxidize hemoglobin to methemoglobin. Apparently, the same

or very closely related systems are operative in the establishment of the reducing conditions and in the formation of oxidizing agents.

Methemoglobin and its reduction product, hemoglobin, may be converted back and forth, one to the other, by the use of an oxidizing reagent, such as ferricyanide, and a reducing reagent, such as sodium hydrosulfite. The action of living pneumococci or of sterile pneumococcus extracts is likewise reversible. By regulation of the tension of molecular oxygen in a reaction mixture of blood and pneumococcus cellular substances, the equilibrium of the reversible reaction Hb$-$e$\rightleftarrows$MetHb may be shifted in either direction without introducing into the system any reagent other than molecular oxygen. There is a limit, however, to the number of times which the reactions may be reversed with the same material. With these relations in mind, it is obvious that the oxygen tension as well as the concentration of bacterial substances is of importance in the determination of the direction and extent of the blood pigment change. These conditions are especially important in case the initial supply of molecular oxygen is limited, since after the consumption of molecular oxygen in a closed system, the pneumococci or the sterile bacterial extracts rapidly establish their own reducing conditions. In such cases, the initial supply of molecular oxygen rather than the concentration of bacterial substances determines the extent of change in the blood pigments.

That oxygen-dissociated hemoglobin, and not oxyhemoglobin, is the substance actually oxidized in the methemoglobin reaction, was suggested in the early literature. Definite proof was furnished by Conant's electrochemical study. That this fact holds true in the case of methemoglobin formation by Pneumococcus was suggested by the experiments of Cole (13) and demonstrated more recently by Neill and Avery (67). To judge from experiments to be presented in another communication, hemoglobin after combination with molecular oxygen or carbon monoxide presents considerable resistance to the oxidizing agents formed by Pneumococcus. It is interesting to recall in this connection the observations of Laidlaw (57) on the apparent stabilizing effect of the addition of molecular oxygen to the iron of the hemoglobin molecule as revealed by the relative ease with which the iron-free hematoporphyrin molecule can be split off from deoxygenated hemoglobin.

The actual reduction of methemoglobin to hemoglobin by sterile animal tissue is demonstrated by the quantitative data of the preceding experiments. The ability of animal tissue to reduce methemoglobin assumes considerable importance in relation to the accumulation of methemoglobin in the blood of patients suffering from infections with Pneumococcus or from intoxication with methemoglobin-forming drugs. It is possible that considerable methemoglobin can actually be formed in the body without a detectable amount accumulating in the blood stream, since, if the tissues are not overtaxed, their inherent reducing activity may reconvert a certain amount of methemoglobin to hemoglobin. On the other hand, if overwhelming amounts are formed, the tissues of even a normal animal may be overtaxed and hence be unable to reduce all the methemoglobin, in which case some of the pigment may be eliminated as such or be destroyed to other products.[1] Moreover, the same deleterious agent which is responsible for the methemoglobin formation may weaken the reducing power of the tissues. Whether methemoglobin accumulates in the animal blood depends apparently upon the balance between the forces producing methemoglobin and the inherent ability of the animal tissues, especially the liver, to reduce methemoglobin to hemoglobin. The accumulation of large amounts of methemoglobin in the circulating blood is probably an indication not only of the formation of large amounts of this pigment but of a "poisoning" of the normal reducing mechanism of the animal's tissues.

Methemoglobin is so readily formed in sterile blood *in vitro* that there must be some mechanism in the body either to prevent its formation or to reduce it back to hemoglobin. The long recognized reducing action of animal tissues as illustrated by Ehrlich's (18) classical experiments seems to afford at least a partial explanation of the fact that methemoglobin does not accumulate in the blood stream of normal animals.

The reversible relations between oxyhemoglobin, hemoglobin, and methemoglobin, which have been demonstrated in the above experiments with Pneumococcus, suggest the possibility of using these blood pigments as indicators of the oxygen-consuming and oxidizing-reducing activities of bacterial cultures and of sterile culture media. However, the application of the blood pigments as indicators is limited

by the fact that many oxidations are catalyzed or accelerated by the presence of even small amounts of these pigments (Schönbein (1857, 1858); His (1856); Robinson (1924); Dakin (1922); Kastle (1909)); and hence, their addition to a system immediately changes its potential oxidizing-reducing powers. (Similar objections may also be raised to the use of methylene blue as an indicator (66).)

SUMMARY.

The Pneumococcus is capable of oxidizing hemoglobin to methemoglobin and of reducing the latter to hemoglobin again. Both the living organism and sterile extracts will accomplish the changes; and like systems seem to be involved in them. The type of reaction induced is determined by the presence or absence of molecular oxygen.

The oxidation and reduction of the blood pigments are reversible processes. The equilibrium between hemoglobin and methemoglobin in a mixture of blood and pneumococcus cellular substances may be shifted in either direction at will by regulation of the oxygen tension.

In the present paper quantitative evidence is presented of the reduction of methemoglobin to hemoglobin by sterile animal tissues. The possible relations of this activity to the disappearance of methemoglobin from the blood stream of the living animal are discussed.

BIBLIOGRAPHY.

1. Araki, T., *Z. physiol. Chem.*, 1890, xiv, 405.
2. Avery, O. T., and Cullen, G. E., 1921, unpublished observations.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
4. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 543.
5. Barcroft, J., The respiratory function of the blood, Cambridge, 1914.
6. Blake, F. G., *J. Exp. Med.*, 1916, xxiv, 315.
7. Bohr, C., *Centr. Physiol.*, 1890, iv, 249.
8. Boxer, S., *Centr. Bakt., I. Abt., Orig.*, 1906, xl, 591.
9. Boycott, A. E., *J. Hyg.*, 1911, xi, 443.
10. Clark, W. M., *J. Washington Acad. Sc.*, 1920, x, 255.
11. Clark, W. M., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 443.
12. Clark, W. M., and Cohen, B., *Abstr. Bact.*, 1922, vi, 3.
13. Cole, R., *J. Exp. Med.*, 1914, xx, 363.
14. Conant, J. B., *J. Biol. Chem.*, 1923, lvii, 401.
15. Dakin, H. D., Oxidations and reductions in the animal body, London, New York, Toronto, Bombay, Calcutta, and Madras, 2nd edition, 1922, 9.
16. Dennig, A., *Deutsch. Arch. klin. Med.*, 1899–1900, lxv, 524.

17. Dittrich, P., *Arch. exp. Path. u. Pharmakol.*, 1891–92, xxix, 247, 251.
18. Erhlich, P., Das Sauerstoff-Bedürfniss des Organismus, Berlin, 1885.
19. Ellinger, P., *Z. physiol. Chem.*, 1920, cxi, 86.
20. Emmerich and Tsuboi, *Vrach*, 1893, No. 25, 722, cited by Filipowski (1895).
21. Filehne, W., *Arch. exp. Path. u. Pharmakol.*, 1878, ix, 329.
22. Filipowski, J., *Arch. sc. St. Petersburg*, 1895, iii, 1.
23. Gallagher, P. H., *Biochem. J.*, 1923, xvii, 515.
24. Gamgee, A., *Phil. Tr. Roy. Soc. London*, 1868, clviii, 589.
25. Gilbert, A., and Fournier, L., *Compt. rend. Soc. biol.*, 1896, xlviii, 2.
26. Gillespie, L. J., *Soil Sc.*, 1920, ix, 199.
27. Grüter, W., *Centr. Bakt., 1. Abt., Orig.*, 1909, l, 241.
28. Haldane, J., *J. Physiol.*, 1897–98, xxii, 298.
29. Haldane, J., Makgill, R. H., and Mavrogordato, A. E., *J. Physiol.*, 1897, xxi, 171.
30. Harden, A., and Zilva, S. S., *Biochem. J.*, 1915, ix, 379.
31. Harrop, G. A., Jr., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 10.
32. Hasselbalch, K. A., *Biochem. Z.*, 1909, xix, 435.
33. Hayem, G., *Compt. rend. Acad.*, 1884, xcviii, 580.
34. Hayem, G., *Compt. rend. Acad.*, 1886, cii, 698.
35. Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.
36. Hoppe-Seyler, F., *Centr. med. Wissensch.*, 1864, ii, 817.
37. Hoppe-Seyler, F., *Z. physiol. Chem.*, 1877–78, i, 396.
38. Hoppe-Seyler, F., Handbuch der physiologisch- und pathologisch-chemischen Analyse für Aerzte und Studierende, Berlin, 6th edition, 1893, 277, 280, 281.
39. Hastings, A. B., Neill, J. M., Morgan, H. J., and Binger, C. A. L., *J. Clin. Inv.*, 1924, i, 25.
40. Harington, C. R., and Van Slyke, D. D., *J. Biol. Chem.*, 1924, lxi, 575.
41. Henninger, *Compt. rend. Soc. biol.*, 1882, 711, referred to in *Jahresb. Fortschr. Thierchem.*, 1882, xii, 121.
42. Heynsius, A., *J. Anat. and Physiol.*, 1868, iii, 123.
43. His, W., *Virchows Arch. path. Anat.*, 1856, x, 483.
44. Heubner, W., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 241.
45. Hüfner, G., *Arch. Physiol.*, 1894, 130.
46. Hüfner, G., and Otto, J., *Z. physiol. Chem.*, 1882–83, vii, 65.
47. Hüfner, G., and Külz, R., *Z. physiol. Chem.*, 1882–83, vii, 366.
48. Jäderholm, A., *Z. Biol.*, 1877, xiii, 193.
49. Jäderholm, A., *Z. Biol.*, 1880, xvi, 1.
50. Kämmerer, H., *Arch. exp. Path. u. Pharmakol.*, 1920, lxxxviii, 247.
51. Kastle, J. H., *Bull. Hyg. Lab., U. S. P. H., No. 51*, 1909.
52. Kobert, R., Lehrbuch der Intoxikationen, Stuttgart, 1893, 70.
53. Kobert, R., *Arch. ges. Physiol.*, 1900, lxxxii, 603.
54. Kobert, R., Lehrbuch der Intoxikationen, Stuttgart, 2nd edition, 1902, i, 97.
55. Kobert, R., Kompendium der praktischen Toxikologie, Stuttgart, 5th edition, 1912, 200–204.

56. Küster, W., *Z. physiol. Chem.*, 1910, lxvi, 165.
57. Laidlaw, P. P., *J. Physiol.*, 1904, xxxi, 464.
58. Lankester, E. R., *Quart. J. Micr. Sc.*, 1870, x, 402.
59. Lewin, L., *Arch. exp. Path. u. Pharmakol.*, 1894–95, xxxv, 401.
60. Lundsgaard, C., and Van Slyke, D. D., Cyanosis, Medicine Monographs, Baltimore, 1923.
61. Marchand, F., *Arch. exp. Path. u. Pharmakol.*, 1887, xxiii, 273.
62. McLeod, J. W., and Gordon, J., *J. Path. and Bact.*, 1922, xxv, 139; *Biochem. J.*, 1922, xvi, 499.
63. McLeod, J. W., Gordon, J., and Pyrah, L. N., *J. Path. and Bact.*, 1923, xxvi, 127.
64. Medigreceanu, F., *J. Exp. Med.*, 1914, xix, 303.
65. Morgan, H. J., and Neill, J. M., *J. Exp. Med.*, 1924, xl, 269.
66. Meyerhof, O., *Arch. ges. Physiol.*, 1912–13, cxlix, 250; 1917, clxix, 87.
67. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xxxix, 757.
68. Neill, J. M., and Hastings, A. B., *J. Biol. Chem.*, 1925, lxiii (in press).
69. Otto, J. G., *Biol. Zentr.*, 1884–85, iv, 732.
70. Peabody, F. W., *J. Exp. Med.*, 1913, xviii, 1.
71. Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.
72. Preyer, W., *Arch. ges. Physiol.*, 1868, i, 395.
73. Rieke, H., *Centr. Bakt., 1. Abt., Orig.*, 1904, xxxvi, 321.
74. von Reinbold, B., *Z. physiol. Chem.*, 1913, lxxxv, 250.
75. Robinson, M. E., *Biochem. J.*, 1924, xviii, 255.
76. Rous, P., *Physiol. Rev.*, 1923, iii, 75.
77. Rymowitsch, F., *Centr. Bakt., 1. Abt., Orig.*, 1902, xxxii, 385.
78. Saarbach, L., *Arch. ges. Physiol.*, 1882, xxviii, 387.
79. Schmitt, *Rev. méd. est*, 1892, Sept. 15, referred to in *Jahresb. Fortschr. Thierchem.*, 1892, xxii, 88.
80. Schnabel, A., *Z. Hyg. u. Infectionskrankh.*, 1921, xciii, 175.
81. Schönbein, C. F., *Verhandl. Naturf. Ges. Basel*, 1857, i, 467, cited by Kastle (51).
82. Schönbein, C. F., *Verhandl. Naturf. Ges. Basel*, 1858, ii, 9, referred to in *Canstatt's Jahresb.*, 1858, 30.
83. Schottmüller, H., *Münch. med. Woch.*, 1903, l, 849, 909.
84. Sorby, H. C., *Quart. J. Sc.*, 1865, 198, 209.
85. Sorby, H. C., *Quart. J. Micr. Sc.*, 1870, x, 400.
86. Sorby, H. C., *Month. Micr. J.*, 1871, vi, 9.
87. Stadie, W. C., *J. Exp. Med.*, 1919, xxx, 215.
88. Stadie, W. C., *J. Exp. Med.*, 1921, xxxiii, 627.
89. Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.
90. Stokes, G. G., *Phil. Mag.*, 1864, xxviii, series 4, 391; *Proc. Roy. Soc. London*, 1863–64, xiii, 357.
91. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.
92. Weyl, T., and von Anrep, B., *Arch. Physiol.*, 1880, 175.
93. von Zeynek, R., *Arch. Physiol.*, 1899, 460.